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PRIMAQUINE: MODES OF ACTION AND MECHANISMS OF DRUG RESISTANCE.(U)
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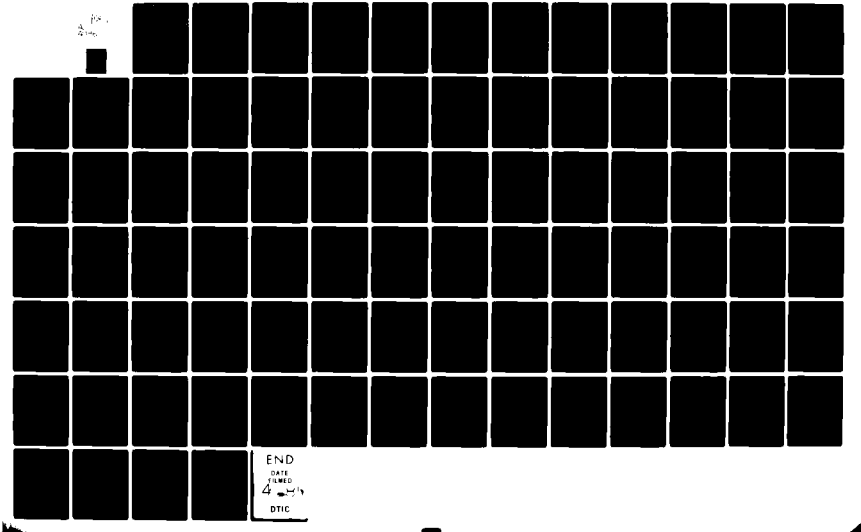

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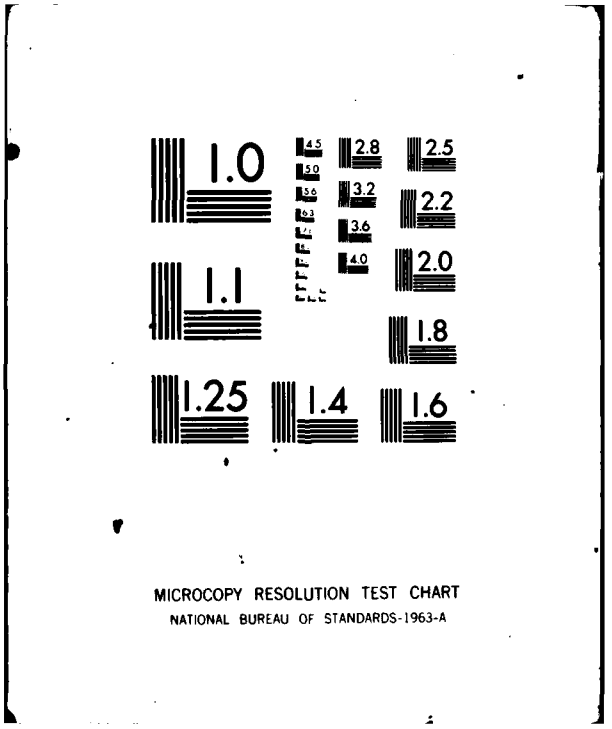
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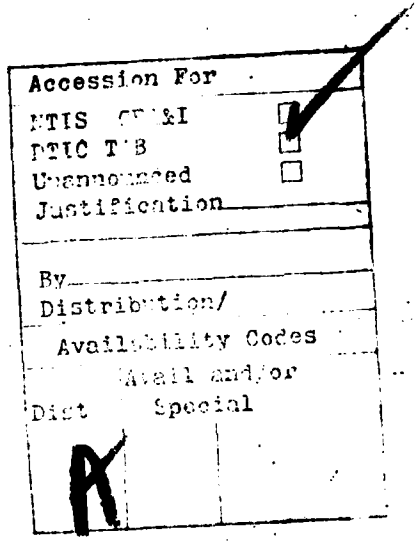
20. Abstract

Tetrahymena pyriformis, a ciliate protozoan, has been used to study the mechanism of action of the antimalarial drug, primaquine. These studies have been performed using both intact *T. pyriformis* and several subcellular fractions and organelles from this organism. *In vivo* studies were utilized to determine the effects of the drug on the uptake and incorporation of macromolecular precursors by the cells. The *in vitro* preparations made it possible to study the effects of the drug on *in vitro* protein synthesis, nucleic acid synthesis *in vitro* and in isolated nuclei, *in vitro* lipid synthesis, and membrane transport and permeability. In addition, a procedure was developed to differentiate DNA and RNA on filter paper discs.

Studies were also performed on a primaquine-resistant strain of *T. pyriformis*. The studies completed were the initial characterization of the strain.



Abstract

Tetrahymena pyriformis, a ciliate protozoan, has been used to study the mechanism of action of the antimalarial drug, primaquine. These studies have been performed using both intact *T. pyriformis* and several subcellular fractions and organelles from this organism. *In vivo* studies were utilized to determine the effects of the drug on the uptake and incorporation of macromolecular precursors by the cells. The *in vitro* preparations made it possible to study the effects of the drug on *in vitro* protein synthesis, nucleic acid synthesis *in vitro* and in isolated nuclei, *in vitro* lipid synthesis, and membrane transport and permeability. In addition, a procedure was developed to differentiate DNA and RNA on filter paper discs.

Studies were also performed on a primaquine-resistant strain of *T. pyriformis*. The studies completed were the initial characterization of the strain.

Table of Contents

I. The effects of primaquine on the uptake and incorporation of macromolecular precursors by synchronized Tetrahymena pyriformis:

A. Introduction and Results: In order to determine the optimal concentrations of primaquine for studying precursor uptake and incorporation, a dose-response for inhibition of cell division was first determined. Table 1 shows the effects of primaquine on synchronized cell division in T. pyriformis. Division normally occurs approximately 80 min. after the end of the synchronizing heat treatment (EHT); however, in this experiment cell counts were made at EHT plus 120 min. in order to distinguish blockade of cell division from a delay in cell division. Primaquine produced 89% inhibition at a concentration of 2.7×10^{-4}M, and this drug level was used in all subsequent experiments. Complete inhibition of cell division was observed with double the above concentration, whereas two-thirds that level of primaquine produced approximately one-half the inhibition.

Primaquine was then examined to determine its effects on the uptake and incorporation of ^{14}C-thymidine, ^{14}C-uridine, and ^{14}C-amino acids. Figure 1 illustrates the effects of primaquine and nalidixic acid, a known inhibitor of DNA synthesis (1), on thymidine incorporation. Primaquine markedly inhibited this process (67.9%) during the 80 min. period after EHT, with essentially no incorporation of thymidine occurring after 40 min. of incubation. Nalidixic acid inhibited DNA synthesis 51.7%. Table 2 compares the effects of primaquine and nalidixic acid at 80 min. after EHT on uptake and incorporation of thymidine. Both uptake and incorporation were blocked by nalidixic acid; however, the inhibition of incorporation (51.7%) was considerably greater than the inhibition of uptake (33.2%). Primaquine was observed to markedly inhibit uptake (61.6%) as well as incorporation (67.9%).

Figure 2 illustrates the effect of primaquine and actinomycin D, an inhibitor of DNA directed RNA synthesis (2), on the incorporation of uridine. Both drugs produced a marked inhibition. Table 3 compares the effect of the drugs at 80 min. after EHT on uptake and incorporation of uridine. Actinomycin D markedly inhibited incorporation (85.4%) while showing considerably less inhibitory effect on uptake (42.6%). Primaquine markedly inhibited both uptake (82.5%) and incorporation (87.5%).

Figure 3 illustrates the effect of primaquine and cycloheximide, a known inhibitor of protein synthesis (3), on the incorporation of amino acids. Both drugs inhibited amino acid incorporation, the greater effect being observed with cycloheximide. The comparison of uptake and incorporation at 80 min. after EHT is shown in Table 4. Cycloheximide was observed to inhibit incorporation (95.5%) to a much greater degree than uptake (48.9%) of amino acids, whereas there was no significant ($P>0.05$) difference between uptake (57.8%) and incorporation (57.7%) in cells treated with primaquine.

To investigate more accurately the effects of primaquine on the uptake of macromolecular precursors, it was necessary to block the synthesis of any one macromolecule, and then examine the effect of the drug on the uptake of the appropriate precursor. In this way it was possible to examine the effect of primaquine on the uptake of precursors (^{14}C-thymidine, ^{14}C-uridine, and ^{14}C-amino acids) independently of incorporation. Figure 4 illustrates the effect of primaquine on the uptake of thymidine. This experiment was performed by incubating the cells with nalidixic acid for the first 20 min. following EHT. This effectively blocked DNA synthesis for the remainder of the experiment (less than 10% of the thymidine taken up was incorporated) but still allowed considerable uptake of thymidine (Table 5). Samples were then assayed for uptake of thymidine from EHT plus 20 min until the time that synchronized cell division normally occurs (EHT plus 80 min.). Primaquine slightly inhibited uptake for the first 30 min., followed by an efflux of the precursors that had entered. At 60 min., the degree of inhibition was 69.2%. During this experiment, primaquine did not significantly change the amount of thymidine incorporation as compared with the control (nalidixic acid only).

Figure 5 illustrates an experiment similar to the above, except that the uptake of uridine was studied. In this experiment, RNA synthesis was blocked (less than 10% of the uridine taken up was incorporated -- Table 5) by incubating the cells with actinomycin D for the first 20 min. following EHT. During the next 60 min., primaquine markedly inhibited uridine uptake (66.5%). Again, primaquine did not significantly alter the residual precursor incorporation as compared with the control (actinomycin D only).

Figure 6 illustrates the effects of primaquine on uptake of amino acids by cell cultures in which protein synthesis had been blocked with cycloheximide (approximately 5% of the amino acids taken up were incorporated -- Table 5). Primaquine was observed to inhibit amino acid uptake (26.8%) without significantly inhibiting the residual amino acid incorporation.

B. Discussion: Primaquine was found to inhibit the incorporation of precursors into DNA, RNA, and protein (Figures 1-3). These results could be explained by inhibition of uptake of the macromolecular precursors, and this was studied by two means. The first was by comparing uptake and incorporation of precursors in the presence of primaquine, and these values were compared to those observed with a known inhibitor of synthesis. The second procedure was to determine the effect of primaquine on uptake of precursors while incorporation was blocked with the known inhibitors. This procedure made it possible to examine precursor uptake under conditions where they could not be utilized, since utilization (incorporation) alone should affect uptake by allowing replenishment of the intracellular precursor pools with exogenous precursors.

Nalidixic acid was found to inhibit incoporation of ^{14}C-thymidine to a considerably greater degree than uptake (Table 2). Since this compound directly inhibits DNA synthesis, it appears that a drug with this as the primary action will inhibit the uptake of thymidine much less than its incorporation. The inhibition of uptake which is observed with nalidixic acid, however, would be expected since inhibition of precursor utilization (i.e., DNA synthesis) from the intracellular pool should also block replenishment of the pool with exogenous precursors. Primaquine, however, produced more inhibition of uptake than did nalidixic acid, and in this case the inhibition was nearly the same as the inhibition of incorporation. Therefore, precursor incorporation appears to be limited by uptake in the presence of primaquine, and this indicates that inhibition of precursor uptake is one of the actions of this drug. To investigate this possibility further, the effect of primaquine on thymidine uptake was determined with cells in which DNA synthesis was blocked with nalidixic acid.These results (Figure 4) substantiate this conclusion since inhibition of uptake was observed.

The effect of actinomycin D on uptake and incorporation of ^{14}C-uridine was that which would be expected of a compound that directly inhibits RNA synthesis, incorporation being inhibited to a much greater extent than uptake (Table 3). Similar reasoning to that described for inhibition of thymidine uptake by nalidixic acid would apply to the inhibition of uridine uptake by actinomycin D. The effects of primaquine on uptake and incorporation of thymidine, thus indicating an inhibitory effect on precursor uptake. This action was substantiated by showing that primaquine produced a marked inhibition of uridine uptake by cells in which RNA synthesis was blocked (Figure 5).

The results illustrating the effects of primaquine on the uptake of thymidine and uridine in the absence of macromolecular synthesis (Figures 4 and 5) show a lag before the maximum inhibitory effects of this drug are observed. In Figure 5, a plateau is observed after 20 to 30 min. of incubation with primaquine. The time which it takes to attain this plateau may represent the time required for the drug to reach effective inhibitory levels at its site of action, which may be the precursor transport mechanisms. The efflux of thymidine from cells treated with primaquine (Figure 4) probably reflects an inability of the cells to maintain their intracellular pools of this precursor, and the lag of 30 to 45 min. before this effect is observed may also represent the time required for the drug to reach an effective concentration at its site of action.

Cycloheximide was shown to inhibit incorporation to a much greater extent than uptake of amino acids (Table 4), a result which would be expected of a compound which directly inhibits protein synthesis. With primaquine, however, if the percentage inhibition of incorporation was compared with that of uptake, no significant difference was observed ($P>0.05$). The results presented in Figure 6

demonstrate inhibition of amino acid uptake by primaquine, and this substantiates the conclusion that one of the effects of this drug is inhibition of amino acid uptake.

C. Conclusion: The results presented above indicate that primaquine inhibits uptake of thymidine, uridine, and amino acids, and this appears to be one of the mechanisms of this drug for blocking incorporation of the precursors into DNA, RNA, and protein respectively.

II. The effects of primaquine on in vitro protein synthesis:

A. Results: The effects of primaquine were studied in a crude cell-free protein synthesizing system isolated from Tetrahymena pyriformis. The cell-free system has previously been characterized in our laboratories (4). The concentrations of primaquine used were those which inhibited cell division 89% (2.7×10^{-4}M) and 100% (5.4×10^{-4}M). The lower drug concentration inhibited in vitro protein synthesis 4%, while the level which completely blocks cell division produced only 6% inhibition.

Another possible loci for inhibition of protein synthesis in vivo would be at the level of the transfer RNA (tRNA) methylases. These enzymes have been shown to control the rate of protein synthesis by altering the functional capacity of the tRNA (5,6). Preliminary work has shown that these enzymes do exist in Tetrahymena, and we have also partially characterized the enzymes. The tRNA methylases were prepared from extracts of Tetrahymena pyriformis GL by precipitation at pH 5.0. The enzyme activity of exponential cells were not inhibited by nicotinamide (Table 6), a known inhibitor of tRNA methylases of rat tissues (7). The supernatant from the cell extract after enzyme precipitation, however, did inhibit the methylase activity. K_m values calculated from reciprocal plots were 1.0×10^{-5}M for tRNA (Figure 7).

B. Discussion and conclusion: Our initial results with in vitro protein synthesis indicate that primaquine does not have a direct inhibitory effect on this process. This supports the suggestion that the effect of primaquine on amino acid incorporation in vivo is due to inhibition of amino acid uptake by the cells, and not due to a direct inhibitory effect on the biosynthetic pathway.

III. The effects of primaquine on nucleic acid synthesis in isolated nuclei:

A. Results: Isolated nuclei (8) from Tetrahymena were first characterized as to requirements for DNA synthesis (Table 7). RNA synthesis has previously been characterized by Lee and Scherbaum (9), and similar requirements for this biosynthetic pathway in isolated nuclei were observed by us (Table 8). The effects of primaquine on DNA and RNA syntheses were studied at 3 drug concentrations: 2.7×10^{-4}M (89% inhibition of cell division); 5.4×10^{-4}M (100% inhibition of cell division); and 2.7×10^{-3} M. Results are shown in Tables 9 and 10.

DNA and RNA syntheses were only slightly inhibited (18% and 7% respectively) at the lowest drug concentration studied, whereas double that concentration produced 26% and 14% inhibition. The highest concentration markedly inhibited both DNA (90%) and RNA (51%) syntheses.

B. Discussion and conclusions: These results show that concentrations of primaquine which markedly inhibit cell division and incorporation of precursors into DNA and RNA in vivo (2.7 and 5.4 x 10^{-4}M) have only a slight inhibitory effect on DNA and RNA syntheses in vitro. Therefore, these results substantiate the findings above, that the effect on incorporation of nucleic acid precursors in vivo is due to inhibition of precursor uptake.

IV. The effects of primaquine on DNA and RNA syntheses by solubilized polymerases:

A. Introduction: The above studies (Part I) demonstrated that primaquine blocks the incorporation of nucleic acid precursors (thymidine for DNA, uridine for RNA) by intact cells. However, if nucleic acid synthesis of the cells was blocked by known inhibitors (nalidixic acid for DNA, actinomycin D for RNA), it was observed that primaquine blocked the uptake of the precursors by the cells. These results indicated that inhibition of precursor uptake could account for the inhibition of incorporation. Further studies (Part II) revealed that primaquine, at concentrations which markedly inhibited precursor incorporation by intact cells, had relatively little inhibitory effect on nucleic acid synthesis in nuclei. These results supported the hypothesis that the primary effect of the drug on intact cells was inhibition of precursor uptake, and not direct inhibition of precursor incorporation. To further investigate the direct effects of primaquine on DNA and RNA syntheses, the respective nucleic acid polymerases were isolated from nuclei.

B. Materials and Methods: Solubilized DNA and RNA polymerases were prepared from nuclei which were isolated by the procedure of Lee and Scherbaum (8). Nuclei from 8 ml of packed cells were suspended in 2 ml of a buffer containing 0.1 M Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 10 mM mercaptoethanol, 0.6 mM EDTA, and 30% (v/v) glycerol (TMG buffer). The nuclei were lysed by addition of NaCl and KCl (concentrated solutions) to final concentrations of 0.15 M each. The lysate was centrifuged at 105,000 x g for 1 hr at 0°C. The supernatant was chromatographed on a Sephadex G-100 column (2 x 35 cm) which had been equilibrated with TMG buffer. The enzymes were eluted with TMG buffer and peak fractions were pooled. The RNA polymerase reaction mixture (0.25 ml) contained 0.5 mM ATP, 0.05 mM GTP and CTP, 0.5 µCi (5-^{3}H)-UTP (17.8 µCi/mmole), 50 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM mercaptoethanol, 0.3 mM EDTA, 15% (v/v) glycerol, 1-3 µg protein (enzyme) as determined by the procedure of Lowry et al. (10), and 50 µg native calf thymus DNA. The DNA polymerase, reaction mixture

(0.25 ml) contained 0.5 mM ATP, 0.05 mM dATP, dCTP, and dGTP, 2.5 μCi (methyl-^{3}H)-TTP (11.1 μCi/mmole), 25 mM KCl, 50 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.3 mM EDTA, 15% (v/v) glycerol, 1-3 μg protein (enzyme), and 50 μg native calf thymus DNA. Both assays were incubated for 60 min at 28°C, and precursor incorporation was determined by the procedure of Byfield and Scherbaum (11).

C. Results: Figure 8 shows the Sephadex G-100 elution pattern for the supernatant of the disrupted nuclei which had been centrifuged for 1 hr at 105,000 x g. The first peak, which was within the void volume of the column, contained both the DNA and RNA polymerase activities. Both activities were proportionally similar within the peak. The four fractions containing the most activity were pooled for enzyme characterization and drug testing (fractions 15-18 in Fig. 8).

Tables 11 and 12, respectively, give the requirements for the DNA and RNA polymerase reactions by the solubilized enzymes, and Figure 9 illustrates the time course for both reactions. Maximum DNA synthesis was dependent upon ATP, dATP, dGTP, dCTP, KCl, and native DNA, since omission of any component or replacement of the native DNA with an equal amount of heat-denatured DNA decreased precursor incorporation. These results are consistent with a DNA-dependent DNA polymerase reaction (DNA replication), and are similar to those described by Pearlman and Westergaard (12) except that they observed more activity with denatured DNA than with native DNA. The RNA synthesis reaction by the solubilized enzyme required ATP, GTP, CTP, and Mn^{++} for maximum activity, and in contrast to the work of Kurtz and Pearlman (13), more activity was observed with native than with denatured DNA. Actinomycin D was observed to inhibit RNA synthesis by 61% at 16 μM and 24% at 1.5 μM, and this effect is similar to that observed with *Tetrahymena pyriformis in vivo* (14). These results are consistent with a DNA-dependent RNA polymerase reaction. The rate of both reactions was observed to progressively decrease with time over a 180 min. period (Figure 9).

Figure 10 illustrates the dose-response of the DNA and RNA polymerase reactions to primaquine, and Table 13 shows in more detail the effects of the drug at the concentrations which produce 90% inhibition (lower dose) and 100% inhibition of cell division in synchronized *T. pyriformis*. The ED_{50} values for inhibition of DNA and RNA syntheses by primaquine were 5.2 mM and 2.2 mM; however, the drug had little inhibitory effect on DNA synthesis at the concentrations which block cell division (Table 13).

D. Discussion: At the concentrations which block cell division in *T. pyriformis* (Table 13), the inhibitory effect of primaquine on DNA and RNA syntheses by the solubilized polymerases was considerably less than the inhibition of thymidine and uridine incorporation into nucleic acids by intact cells (Part I). At the lower drug

concentration, which inhibits precursor incorporation by 67.9% (DNA) and 87.5% (RNA) in intact cells, primaquine produced zero (DNA) and 22% (RNA) inhibition of the solubilized polymerase reactions. The higher drug concentrations produced only slightly greater inhibition of the RNA synthesis reaction. Although it is not known for certain whether the same enzyme is responsible for DNA synthesis in vivo and in vitro, or whether the in vitro reaction is measuring DNA replication or repair, these results indicate that primaquine may not inhibit nucleic acid synthesis. Therefore, the drug may possess another mechanism for blocking precursor incoporation by T. pyriformis in vivo, for example, inhibition of precursor uptake. Further support of this hypothesis are the observations that primaquine is not an effective inhibitor of nucleic acid synthesis in nuclei of T. pyriformis (Part III) that this drug binds only poorly to DNA (15), and that primaquine is a specific inhibitor not of nucleic acid but of protein biosynthesis in Bacillus megaterium (16).

V. The effects of primaquine on in vitro lipid synthesis:

A. Introduction: The results of the above in vivo and in vitro studies have indicated that one of the actions of primaquine is inhibition of precursor uptake, which may be due to alteration of membrane transport. Since lipids are an integral part of membranes of cells and cell organelles, it is possible that inhibition of growth, division, and membrane transport of T. pyriformis by primaquine could be due to inhibition of lipid synthesis.

B. Effects of primaquine on lipid synthesis in the crude homogenate:

1. Materials and Methods

(a) Preparation of the crude homogenate for lipid synthesis: T. pyriformis, strain GL, was grown and harvested as described above. For experimental purposes two day old cultures were used to inoculate 200 ml of the proteose-peptone-liver extract (PPL) medium. Cultures were incubated for 12-18 hours at 29°C. The cells were harvested when the population reached approximately 200,000 per ml, washed twice in 0.5% NaCl, resuspended in 10 ml of a buffer containing 0.1 M phosphate buffer, (pH 7.2), 0.25 M sucrose, and 5 mM mercaptoethanol, and the cells disrupted by homogenization in a tissue grinder with a teflon pestle. The homogenate was centrifuged at 250 x g for 5 min at 4°C. The pellet was discarded and the supernatant was removed for assay. This preparation yielded a crude homogenate with a protein content of approximately 2 mg/ml.

(b) Assay for cell-free lipid synthesis in the crude homogenate: Assay for cell-free lipid synthesis was done using a 0.5 ml reaction mixture containing 375-750 µg protein (cell homogenate) as determined by the procedure of Lowry et al. (10), 0.1 M phosphate buffer (pH 7.2), 0.25 M sucrose,

5 mM mercaptoethanol, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 5 mM ATP, 2.5 mM NADH, 0.75 mM coenzyme A and 0.5 µCi 2-^{14}C-acetate (25 µCi/mmole, Calatomic). The reaction mixtures were incubated at 29°C, and samples (0.1 ml) were taken in duplicate at 0 and 60 min. The samples were placed on 2.3 cm filter paper discs and the discs processed by the procedure of Byfield et al. (17) to determine incorporation of acetate into lipids.

(c) Isolation of lipids: Lipids were isolated from the reaction mixtures after 60 min. of incubation by extraction with chloroform:methanol (2:1 v/v) according to the method of Bligh and Dyer (18). Extracted lipids were washed overnight by the method of Folch et al. (19). Classes of lipids were separated and identified on Silica Gel G thin layer plates by developing with a solvent system containing petroleum ether : diethyl-ether : acetic acid (75:25:1 v/v). Phospholipids were separated by developing with a solvent system containing chloroform : acetic acid : methanol : water (75:25:5:2.2 v/v). After developing the plates, the spots were located by exposure to iodine vapors, the stained areas carefully circled with a lead pencil, and following sublimation of the iodine, the spots were scraped directly into scintillation vials. The radioactivity present on each spot was then determined by measurement in a Beckman liquid scintillation counter, model LS 150, using a PPO-toluene (5g/l) scintillation fluid.

2. Results:

(a) Requirements for incorporation of acetate into lipids: Table 14 shows the requirements for lipid synthesis by the crude homogenate. The complete reaction mixture incorporated 4620 CPM/100 µg protein. Omission of ATP nearly abolished the activity (only 1% remaining), and omission of NADH reduced the activity to 39% of the complete system. When coenzyme A was eliminated the relative incorporation of ^{14}C-acetate was reduced to 56% and when Mg^{++} was omitted the relative incorporation was 65%. Addition of NADPH inhibited lipid synthesis by this cell-free system at 2.5 and 10 mM. Figure 11 illustrates the time course of lipid synthesis by the crude homogenate. Incorporation of acetate was nearly maximal after 60 min.

(b) Effect of primaquine on lipid synthesis by the crude homogenate: Table 15 shows the effects of primaquine on lipid synthesis by this system. The two intermediate doses tested were those that normally inhibit division of synchronized cells by approximately 80% and 100%. Primaquine produced significant inhibitory effects ($p < 0.01$) at all concentrations tested.

(c) Characterization of the lipids synthesized by the crude homogenate: The relative amounts of the classes of lipids produced, as determined by thin layer chromatography, are 3.8% triglycerides, 24.7% free fatty acids (FFA), 63.2% phospholipids and 8.3% tetrahymenol (Table 16). Of the phospholipids synthesized, cardiolipin amount to 82.3%, ethanolamine phosphatides, 8.9%; choline phosphatides, 4.8%, and 2-aminoethylphosphonolipids, 4.1% (Table 17). Tables 16 and 17 show the amounts of the individual classes of lipids synthesized by the crude homogenate in the presence and absence of primaquine. At 5.4×10^{-4}M there was 41% inhibition of acetate incorporation into neutral lipids (triglycerides, FFA and tetrahymenol). The inhibition of triglyceride synthesis (51%) was more prominent than inhibition of FFA (42%) or tetrahymenol (31%) synthesis.

C. Effects of primaquine on lipid synthesis in the mitochondrial fraction:

1. Materials and Methods

(a) Preparation of the mitochondrial fraction for lipid synthesis: Mitochondria were prepared by a modification of the procedure of Schwab-Stey et al. (20). Tetrahymena pyriformis was grown, harvested, and washed as described above for the preparation of the crude homogenate. The washed cells were suspended in 10 ml of a buffer containing 0.1 M phosphate (pH 7.2), 0.3 M sucrose, and 10 mM mercaptoethanol (solution L), disrupted by homogenization and centrifuged at 1000 x g for 10 min. The supernatant was then centrifuged at 7000 x g for 10 min. The pellet was resuspended in 10 ml of a Ficoll solution containing 10 gm of Ficoll in 100 ml of solution L and centrifuged at 1000 x g for 10 min. The supernatant containing mitochondria was then washed twice in Ficoll by centrifugation at 7000 x g for 30 min. The final pellet containing a pure mitochondrial preparation was suspended in 10 ml of solution L. This preparation yielded a mitochondrial preparation with a protein content of 1-1.5 mg/ml. The purity of this preparation was then tested using enzyme markers as indicators of possible contamination by other cell organelles. Succinic dehydrogenase was used as an enzyme marker for the presence of mitochondria, acid phosphatase for lysosomal contamination, and isocitrate dehydrogenase for peroxisomal contamination. Determinations for the activity of these enzymes are as described by Levy, et al. (21).

(b) Assay for mitochondrial lipid synthesis: Assay for mitochondrial lipid synthesis was done using a 0.5 ml reaction mixture containing 200-400 µg of protein as determined by the procedure of Lowry (10), 0.1 M phosphate buffer (pH 7.2), 0.3 M sucrose. 10 mM mercaptoethanol,

0.1 mM EDTA, 2.5 mM $MgCl_2$, 10 mM ATP, 0.4 mM NADH, 0.4 mM NADPH, 0.1 mM CoA, and 0.05 µCi of 2-^{14}C-acetate (25 µCi/mmole, Calatomic). The reaction mixtures were incubated at 29°C, the optimal growth temperature for T. pyriformis, and samples (0.1 ml) were taken in duplicate at 0 and 30 min. The samples were placed on filter paper discs and assayed by the filter paper disc procedure as described above.

(c) Isolation of lipids: Lipids synthesized by this mitochondrial fraction were isolated and characterized in a similar manner to that described for the crude homogenate.

2. Results:

(a) Purity of the preparation: The relative activity of succinic dehydrogenase, acid phosphatase and isocitrate dehydrogenase were determined in the supernatants and pellets of the preparative steps for the isolation of mitochondria from T. pyriformis. Table 18 shows the relative activity in percent of these three enzymes. The pellet after the first 7000 x g 10 min. centrifugation was taken as the crude mitochondrial preparation. This pellet was suspended in Ficoll and centrifuged at 1000 x g. The supernatant contained 42% of the mitochondria, 11% of the lysosomes and 26% of the peroxisomes. The final pellet contained 36% of the mitochondria, but only 5% and 3% of the lysosomes and perixosomes respectively.

(b) Requirements for incorporation of acetate into lipids in the mitochondria: Table 19 shows the requirements for lipid synthesis in the mitochondrial fraction. The complete reaction mixture incorporated 6350 CPM/100 µg protein. Omission of ATP or CoA almost abolished the activity (3% remaining for ATP, 4% for CoA). Both NADH and NADPH were required for the system. Omission of NADH reduced the activity to 35% and absence of NADPH reduced activity to 89%. Magnesium ions were also required (9% remaining in its absence), but Mn^{++} was inhibitory to the system at 2.5 and 5.0 mM. Fig. 12 shows the time course of lipid synthesis by this mitochondrial preparation. Linear incorporation was observed for the first 30 min. and maximum incorporation was attained after 60 min. of incubation. Fig. 13 shows the optimal protein concentration for incorporation using the optimal conditions. Linear incorporation was observed with increasing protein concentration up to 600 µg per reaction mixture.

(c) Effect of primaquine on lipid synthesis in the mitochondrial fraction: Table 20 shows the effects of primaquine on in vitro lipid synthesis in the mitochondrial fraction. At

2.7×10^{-4}M and 5.4×10^{-4}M, doses which normally inhibit synchronous cell division by 80% and 100%, lipid synthesis was inhibited by 6.7% and 32% respectively. At 1.1×10^{-3}M, lipid synthesis was inhibited by 55.5%.

(d) Characterization of the lipids synthesized by the mitochondrial fraction, and the effect by primaquine on synthesis of the individual classes of lipids: Tables 21 and 22 show the percentages of the various classes of lipids synthesized by this fraction in the presence and absence of primaquine. The mitochondria produced 0.5% triglycerides, 3% free fatty acids, 24% tetrahymenol and 73% phospholipids. Primaquine at 5.4×10^{-4}M showed a more prominent inhibition of neutral lipid synthesis (43% versus 35% for phospholipids). Ethanolamine phosphatides were the major phospholipids synthesized and all classes of phospholipids were inhibited by primaquine.

D. Effect of primaquine on lipid synthesis in the microsomal fraction:

1. Materials and Methods

(a) Preparation of the microsomal fraction for lipid synthesis: Tetrahymena pyriformis was grown to a population of 200,000/ml in 1000 ml of PPL medium. The cells were harvested, washed and homogenized as described above for the preparation of the mitochondrial fraction. The pooled supernatants from the 1000 x g centrifugation were then centrifuged at 10,000 x g for 30 min, the pellet was discarded and the microsomal fraction was sedimented by centrifugation at 105,000 x g for 60 min. The final microsomal pellet was suspended in 4 ml of solution L. This preparation yielded a microsomal preparation with a protein content of 3-4 mg/ml. The purity of this fraction was tested using succinic dehydrogenase to determine mitochondrial contamination.

(b) Assay for microsomal lipid synthesis: Assay was done using a 0.5 ml reaction mixture containing 0.1 M phosphate buffer (pH 7.2), 0.3 M sucrose, 10 mM mercaptoethanol, 0.8 mM NADH, 1.0 mM CoA, 10 mM ATP, 5 mM Mg^{++} and 0.05 µCi of 2-^{14}C-acetate (25 µCi/mmole, Calatomic). The reaction tube was incubated at 29°C and samples (0.1 ml) were taken in duplicate at 0 and 30 min and assayed as described above.

(c) Lipids synthesized by this fraction were isolated and characterized in a similar manner to that used for the mitochondrial fraction.

2. Results

(a) Purity of the microsomal preparation: The pellets and supernatants from the various steps of the preparative procedure for the microsomal fraction were assayed for succinic dehydrogenase activity (Table 23). The results showed that most of the mitochondria were removed by centrifugation at 1000 x g and 10,000 x g. Calculated on the basis of succinic dehydrogenase activity, the microsomal fraction contained 2% of the mitochondria originally present.

(b) Requirements for the incorporation of acetate into lipids into the microsomal fraction: Table 24 shows the requirements for the incorporation of acetate into lipids in the microsomal fraction. The complete reaction mixture incorporated 990 CPM/100 µg protein. Omission of ATP and CoA abolished the incorporation almost completely. Omission of NADH and Mg^{++} reduced the activity to 22% and 39% respectively. NADPH, Mn^{++} and EDTA were not required by this fraction for the synthesis of lipids. Fig. 14 shows the time course for lipid synthesis in this fraction. The rate of incorporation was linear up to 30 min. and reached maximal incorporation at 60 min. Fig. 15 gives the relationship of protein concentration to the incorporation of acetate into lipids. The optimal concentration of protein per reaction mixture was between 200-600 µg per reaction mixture.

(c) Effect of primaquine on the incorporation of acetate into lipids in the microsomal fraction: Table 25 shows the effects of primaquine on lipid synthesis in the microsomal fraction at four different concentrations. At doses which inhibit synchronous division by 80% and 100%, lipid synthesis was inhibited by 29% and 40% respectively. At 1.1×10^{-3}M, lipid synthesis was inhibited by 52%.

(d) Isolation and characterization of the classes of lipids synthesized in the microsomal fraction and the effects of primaquine at 5.4×10^{-4}M: Tables 26 and 27 show that the lipids synthesized by the microsomal fraction were primarily free fatty acids (58% of total lipids), with 30% of the total being phospholipids. Primaquine at 5.4×10^{-4}M inhibited the production of neutral lipids by 56% and of free fatty acid by 55%. The synthesis of phospholipids was not inhibited by primaquine in the microsomal preparation.

E. Effect of primaquine on lipid synthesis in the soluble cell fraction

1. Material and Methods

(a) Preparation of the soluble cell fraction for lipid synthesis: The procedure for preparing the soluble cell fraction was similar to that for the preparation of the microsomal fraction. The supernatant after the final centrifugation at 105,000 x g was used as the soluble cell fraction. This preparation contained a protein concentration of 7-10 mg/ml.

(b) Assay for the incorporation of acetate into lipids: The assay was performed in a reaction mixture containing 0.1 M phosphate buffer (pH 7.2) 0.3 M sucrose, 10 mM mercaptoethanol, 1 mM EDTA, 2 mM NADH, 2 mM NADPH, 0.4 mM CoA, 4 mM ATP, 4 mM Mn^{++}, 2 mM Mg^{++} and 0.1 µCi of 2-^{14}C-acetate (25 µCi/mmole, Calatomic) in a total volume of 0.5 ml. The amount of protein in each reaction mixture was 1-2 mg. The reaction mixture was incubated at 29°C and samples (0.1 ml) were removed in duplicate at 0 and 90 min. The samples were then assayed by the filter paper disc procedure as described above.

2. Results

(a) Requirements for the incorporation of acetate into lipids in the soluble cell fraction: Table 28 shows the requirements for the incorporation of acetate into lipids by the soluble cell fraction. This fraction required both NADH and NADPH for optimal activity. Omission of either of these cofactors reduced the incorporation to just 5%. Omission of ATP reduced the activity to 1% and omission of CoA reduced activity to 3%. Both Mn^{++} and Mg^{++} were required by the system for maximal synthesis of lipids. EDTA was also required by this fraction. Fig. 16 shows the time course for lipid synthesis in this fraction. Linear incorporation was observed in the first 90 min and maximal incorporation was attained at approximately 120 min. Fig. 17 shows that the optimal protein concentration to use for each reaction sample was between 1-2 mg.

(b) Effects of primaquine on the incorporation of acetate into lipids in the soluble cell fraction: Table 20 shows that primaquine at 2.7×10^{-4}M and 5.4×10^{-4}M inhibited lipid synthesis by 42.8 and 62.5% respectively. At 1.1×10^{-3} M, lipid synthesis was inhibited by 85.2%.

(c) Isolation and characterization of products synthesized in the soluble cell fraction. Tables 30 and 31 show that the neutral lipids synthesized in the soluble cell fraction were

5% triglycerides, 28% FFA, and 34% tetrahymenol. Of the phospholipids synthesized, 44% were ethanolamine phosphatides, 23% choline phosphatides and 33% 2-aminoethylphosphonolipids. Primaquine at 5.4×10^{-4} M showed a 72% inhibition of neutral lipid synthesized, FFA was inhibited by 87%, tetrahymenol by 60% and triglycerides by 63%.

F. Discussion: Subcellular fractions from T. pyriformis have been prepared and characterized for lipid synthesis using ^{14}C-acetate as a precursor for the synthesis of lipids. The classes of lipids synthesized were identified using thin layer chromatography. In our characterization procedure we did not attempt to identify the different fatty acids that were synthesized. However, we did show that all classes of lipids are synthesized by the various subcellular fraction, and the percentage of the various classes of lipids synthesized vary with the fraction studied. The mitochondrial fraction synthesized more phospholipids than either the microsomal or the soluble cell fraction (76% phospholipids for the mitochondrial fraction vs. 30% for the microsomal, and 34% for the soluble cell fraction). However, free fatty acid synthesis is more active in the microsomal and soluble cell fractions. Tetrahymenol, a triterpenoid synthesized exclusively in T. pyriformis is known to be a cholesterol replacement in this organism (22) and this compound is more actively synthesized in the soluble cell fraction.

The effect of primaquine at doses of 1.1×10^{-4}M, 2.7×10^{-4}M, 5.4×10^{-4}M and 1.1×10^{-3}M were studied on the crude homogenate as well as on each of the three subcellular fractions. The results demonstrated that primaquine has a potent inhibitory effect on lipid synthesis. However, the degree of inhibition differed considerably between fractions. This may be due in part to the fact that the products synthesized by these various fractions were different, and that primaquine consistently inhibited neutral lipid synthesis to a greater extent than phospholipid synthesis.

These results show that primaquine, at doses which inhibit cell division and growth, has a potent inhibitory effect on the synthesis of lipids, primary neutral lipids. This effect of primaquine on the synthesis of lipids may contribute to the inhibition of growth and division of this organism.

VI. The effects of primaquine on membrane transport and membrane permeability in T. pyriformis as studies with ^{14}C-amino acids:

A. Introduction: Our results using intact cells (Part I), isolated nuclei (Part III), solubilized nucleic acid polymerases (Part IV) and a cell-free protein synthesizing system (Part II) have indicated that primaquine inhibits the incorporation of precursors for DNA, RNA, and protein by inhibition of precursor uptake by intact cells, and not by direct inhibition of macromolecular synthesis. Two possible mechanisms could explain these results. First, primaquine

could block the active transport of the precursors into the cell, and secondly, the drug could alter the permeability of the cell membrane so that the cells are unable to maintain their intracellular pool of precursors. Both mechanisms have been investigated using a ^{14}C-amino acid mixture (our precursors for measuring protein synthesis). The working hypothesis for investigating membrane transport was to determine the effects of the drug on precursor binding to membrane-bound transport proteins (23, 24). Membrane permeability studies were designed to measure the loss of labeled amino acids from intracellular pools, the pools being labeled following complete inhibition of protein synthesis by cycloheximide.

B. Membrane transport studies

1. Methods and Results: Initial studies were designed to determine the effects of primaquine on the binding of amino acids to cell membrane preparations as a measure of the effect of the drug on substrate binding to membrane transport proteins. T. pyriformis were grown to a population of 200,000/ml, washed free of culture medium, and resuspended in Ryley's phosphate buffer (25). To 10 ml of the cells was added 25 µCi of a ^{14}C-amino acid mixture (algal profile). Aliquots (1.0 ml) were removed at 0, ½, 1, 2, 5, 10, 15, and 30 min. and placed in 3.0 ml of cold ethanol. The cells were sedimented by centrifugation and cell ghosts (cell membrane preparations) were prepared by treatment with 3% Lubrol W at 37°C for 5 min. The ghosts were washed twice with cold 0.5% NaCl and resuspended in this solution. One tenth ml aliquots of the suspensions were placed on 2.3 cm filter paper discs, dried, and counted by liquid scintillation spectrometry to determine binding of amino acids to the cell membrane. The results of these determinations, as well as the results of parallel studies done in the presence of primaquine and cycloheximide (a known inhibitor of protein synthesis - ref. 3), are shown in Figure 18. The control samples showed a rapid association of the ^{14}C-amino acids with the cell ghosts. Primaquine significantly reduced the amount of labeled amino acids associated with the ghosts, and cycloheximide produced a much greater reduction.

The marked reduction in membrane-associated amino acid by cycloheximide indicated that the association may not be a binding to membrane transport proteins, but, in fact, an actual incorporation of the amino acids into membrane associated proteins. To investigate this possibility, duplicate samples of those described above were processed by the filter paper disc procedure (11) to determine incorporation of amino acids into protein. The results (Figure 19) show that only 25% of the ^{14}C-amino acids of the control samples, and less than 10% of those in the samples treated with primaquine and cycloheximide, were incorporated into protein as measured by this procedure.

Due to the equivocal results of the above experiments, we next investigated binding by direct incubation of amino acids with cell ghosts. The cell ghosts were prepared as described above from cells which had been grown under standard conditions the ghosts were then suspended in Ryley's buffer at a concentration of 10^6 ghosts/ml. The ghosts were incubated with 2.5 μCi/ml of the ^{14}C-amino acid mixture, and samples were taken at 0, 2, 5, and 10 min. Reactions were run in the presence and absence of 5 mM ATP to investigate the possibility of an energy-dependent binding. The samples were immediately applied to millipore filters and thoroughly washed with 10 times the volume of Ryley's buffer. The millipore filters were dried and counted by liquid scintillation spectrometry. Neither reaction mixture showed significant binding of amino acids to the cell ghosts.

2. Discussion: A possible explanation for the results presented above is that the association of amino acids with the cell ghosts, as shown by incubation of amino acids with intact cells, represents incorporation of the precursors into membrane-associated proteins via the classical protein synthesis mechanisms. This is suggested by the marked inhibition of this reaction by cycloheximide (Figure 18). However, when the samples were processed by the filter paper disc procedure, only 25% of the radioactivity in the control and less than 10% of radioactivity in the drug treated samples were shown to be incorporated into proteins (Figure 19). These results could be interpreted in two ways. First, all of the amino acids associated with the ghosts may be incorporated into proteins and polypeptides which are an integral part of the cell membrane. In this instance, the explanation for the marked reduction in radioactivity after processing by the filter paper disc procedure could be that a large percentage of the polypeptides are small, and thus washed from the discs during the washing procedure. Secondly, only a portion of the counts are incorporated into protein and the remainder are actually bound to the membrane, possibly in association with membrane transport structures. Recent results by other investigation (26) support the former hypothesis.

   The results of incubation of amino acids with isolated ghosts indicate that binding does not occur under these conditions. A possible explanation is that the preparative procedure for the ghosts, which involves exposure to substances which may destroy secondary, tertiary, and quaternary structures of proteins and/or transport structures, may abolish the binding capacity of the transport systems for the amino acids.

C. Membrane permeability studies:

1. Methods and Results: T. pyriformis were grown to a population of 100,000/ml, synchronized by a cyclic heat treatment washed, and resuspended in Ryley's buffer (25). Cycloheximide was

added to the culture at a final concentration of 5×10^{-6}M, a level which inhibits protein synthesis by 95%. After a 10 min. incubation period, 20 μCi of a ^{14}C-amino acid mixture was added to 20 ml of the cell suspension and the incubation was continued for an additional 20 min. The cells were then washed with Ryley's buffer and resuspended to the original volume with Ryley's buffer containing 5×10^{-6} M cycloheximide. This procedure effectively labeled the intracellular amino acid pool (1415 CPM/0.1 ml cell suspension). Ten ml aliquots of the cell suspension were pipetted into 50 ml Erlenmeyer flasks, one containing primaquine at 2.7×10^{-4} M (the concentration which inhibits cell division by 90%), and the other a control flask without drug. At 10-15 min. intervals for 1 hr, 1.5 ml aliquots were removed and the cells sedimented by centrifugation at 250 x g for 30 sec. Samples of the supernatant (0.1 ml) were placed on 2.3 cm filter paper discs, dried, and counted by liquid scintillation spectrometry. The amount of amino acids appearing in the supernatant represents that which has diffused from the intracellular pool. These results are shown in Figure 20. During the 60 min. incubation period, the control sample was observed to release 50 CPM/0.1 ml of the cell suspension, or 3.5% of the intracellular ^{14}C-amino acids. The samples treated with primaquine released 115 CPM/0.1 ml of the cell suspension, or 8.1% of the intracellular ^{14}C-amino acids. Samples were also taken at the beginning and end of the 60 min. incubation period and processed to determine incorporation of amino acids into macromolecules (11). Neither the control nor the primaquine treated sample showed significant incorporation.

2. Discussion: The results presented above indicate that primaquine does not alter membrane permeability with resultant loss of protein precursors from intracellular pools. There is, however, a small loss of precursors from normal cells (3.5%), and although the amount of loss is more than double in the presence of primaquine (8.1%), this amount still represents only a very small proportion of the intracellular pool. The results demonstrating that the amino acids are not incorporated into protein during the 60 min. incubation period indicate that these precursors remain in the form of free amino acids. This is due to the observation that incorporation into protein, or even formation of amino acyl-transfer RNA, would be detected as incorporation by the processing procedure (11).

VII. The effects of primaquine on membrane transport systems as studied with amino acid and carbohydrate analogues:

1. Introduction: Alpha-aminoisobutyric acid (AIB), the most widely used amino acid analogue for transport studies, cycloleucine, an amino acid which is not incorporated into protein (27), and 2-deoxyglucose, a non-metabolised sugar analogue (28), have been used as non-metabolized substrates to study membrane transport in the presence and absence of primaquine in T. pyriformis.

2. Materials and Methods: Tetrahymena pyriformis was grown to a population of 200,000/ml in PPL medium, washed, and resuspended in Ryley's buffer. The cells were incubated with 0.25 μCi/ml of ^{14}C-labeled precursor and uptake was determined at intervals between 0 and 120 min. by withdrawing 1.5 ml aliquots of the cells, sedimenting the cells by centrifugation at 500 x g for 30 sec., and determining the disappearance of the labeled substrates from the supernatant. This determination was done by placing 0.1 ml samples of the supernatants on 2.3 cm filter paper discs, drying the discs at 60°C, and counting the radioactivity by liquid scintillation spectrometry. The effect of primaquine at 5.4×10^{-4} M on the uptake of these non-metabolized precursors was compared with control cells.

3. Results: We have examined the uptake of cycloleucine, AIB, and 2-deoxy-D-glucose by T. pyriformis. Fig. 21-23 show the uptake of these precursors by normal log phase cells and the effect of primaquine on this process. Cycloleucine was taken up to a greater extent (> 1000 CPM at 120 min.) than either AIB (< 400 CPM at 120 min.) or 2-deoxy-D-glucose (> 400 CPM at 150 min.). Primaquine at 5.4×10^{-4} M showed an initial stimulatory effect for the first 30 min. followed by inhibition of uptake of all three compounds. To further examine this initial stimulatory effect, cells were incubated with primaquine for 30 min. followed by the addition of ^{14}C-cycloleucine. Fig. 24 shows that pre-incubation with primaquine produced continuous inhibition throughout the incubation period.

4. Discussion: Primaquine was shown to stimulate the uptake of all precursors studied, for the first 30 min. of incubation if the drug and precursor were added at the same time. After 30 min., the uptake of each precursor was completely inhibited. Similar results were observed for the effects of primaquine on the uptake of nucleic acid precursors (Part I). Preincubation with the drug for 30 min. before addition of the precursor (cycloleucine) abolished the stimulating effect, and only inhibition of uptake was observed. A possible explanation of these results is that primaquine initially (e.g. for 30 min) increased the permeability of the cell membrane to the precursor, and they enter more rapidly by simple diffusion. However, once the intracellular concentration is equal to the extracellular concentration of precursor, further uptake of the precursor (e.g. by active transport) is inhibited by primaquine.

VIII. Differentiation of DNA and RNA on filter paper discs:

A. Introduction: In our investigations of DNA and RNA syntheses in T. pyriformis, we have found it necessary to develop a simple procedure for differentiating between these two nucleic acids. Two factors made it necessary for the development of this procedure. First, procedures requiring long incubation with

specific precursors for DNA (e.g. thymidine) or RNA (e.g. uridine) have shown that the precursors can be metabolized to forms which are incorporated into both nucleic acids. Secondly, some precursors (e.g. orotic acid or other free purine or pyrimidine bases) are normally incorporated into both DNA and RNA even with short incubation procedures. We have, therefore, developed a procedure for differentiating between DNA and RNA using a modification of the filter paper disc procedure for assay of nucleic acid synthesis (11).

B. Summary of the procedure: Cells which have been incubated in the presence of a ^{14}C-labeled nucleic acid precursor are placed, in 0.1 ml aliquots, on 2.3 cm Whatman 3MM filter paper discs. The discs are placed, while still wet, into cold 5% trichloroacetic acid (TCA). The discs are then washed by the standard filter paper disc procedure of Byfield and Scherbaum (11). This procedure consists of 3 successive washes for 15 min. each in cold 5% TCA, followed by 2 washes for 5 min. each in a cold ethanol-ether mixture (1:1), followed by 2 washes for 5 min. each in cold ether. The discs are dried in air, and the total radioactivity incorporated into nucleic acids determined by liquid scintillation spectrometry. The discs are subsequently removed from the vials, rinsed 3 times in acetone to remove the scintillation fluor and dried. The discs are then impaled on stainless steel pins, and to each is added 0.2 ml of 0.5 N NaOH. The discs are placed in a humidity chamber at 37°C for 90 min, after which they are processed by the washing procedure as before. This procedure quantitatively removes the RNA. The discs are recounted by liquid scintillation spectrometry to determine the amount of RNA in the sample (the radioactivity removed by NaOH hydrolysis plus a correction factor - see below), after which the discs are removed from the vials, washed in acetone and dried. The discs are treated with 5% TCA for 30 min. at 90°C, followed by the washing procedure as before. This process quantitatively removed the DNA which remained on the discs. The discs are recounted to determine the amount of DNA in the sample (the radioactivity removed by the hot 5% TCA hydrolysis plus a correction factor - see below). Remaining radioactivity is assumed to be unidentified compounds.

C. Supporting data: For experiments to characterize the hydrolysis procedure, we utilized DNA and RNA which were prepared by 2 different methods. The first method was by pulse labeling cells with specific precursors for the nucleic acids. T. pyriformis were grown to a population of 200,000/ml in proteose-peptone-liver extract medium, washed with Ryley's buffer, and resuspended in this buffer to the original population. To one aliquot of the cell suspension was added ^{14}C-thymidine (2.5 µCi/ml) to specifically label DNA. After a 10 min. incubation, 0.1 ml aliquots were placed on 2.3 cm filter paper discs and the discs placed in cold 5% TCA. Another aliquot of the cell suspension was incubated with ^{14}C-uridine (2.5 µCi/ml) in a similar manner to label the RNA.

The second procedure for preparing the nucleic acids was by labeling culture of cells with either ^{14}C-thymidine or ^{14}C-uridine, followed by isolation of DNA and RNA from the cells. T. pyriformis were grown to a population of 150,000/ml in 1000 ml of proteose-peptone-liver extract medium. Twenty-five µCi of ^{14}C-thymidine was added and the cells were incubated for 3 hr. A similar incubation was done with 25 µCi of ^{14}C-uridine. The cells were collected after the incubation period by centrifugation, and DNA and RNA were isolated by the procedure of Rudin and Albertsson (29). This isolation procedure is based on the partition of the macromolecules in a polymer two-phase system of polyethylene glycol and dextran. Filter paper discs were then prepared using either 0.1 ml aliquots of the solution of isolated DNA, or 0.1 ml aliquots of the solution of isolated RNA. The discs prepared by this procedure were then washed by the standard filter paper disc washing procedure, dried, and counted by liquid scintillation spectrometry. After counting, the discs were washed with acetone and dried in preparation for the NaOH hydrolysis procedure.

Figure 25 illustrates the time course for hydrolysis of RNA prepared by the pulse label procedure, and Figure 26 illustrates the results with isolated RNA. The discs, after being impaled on stainless steel pins, were incubated in a humidity chamber at 37°C with 0.2 ml of NaOH at concentrations of 0.25 N, 0.5 N, or 1.0 N. After incubation for the times indicated, discs (in duplicate) were removed from the humidity chamber and placed in cold 5% TCA. At the completion of the 2-1/2 hr. hydrolysis experiments, all discs were washed simultaneously by the standard washing procedure, dried, and counted by liquid scintillation spectrometry. The hydrolysis curves were similar for the RNA prepared by both procedures. At 0.25 N NaOH, maximum hydrolysis was achieved at 90 to 120 min., with 0.5 N NaOH the maximum hydrolysis was observed at 60 to 90 min., and with 1.0 N NaOH, the maximum hydrolysis was at 30 to 60 min. Hydrolysis with 0.5 N NaOH for 90 min. was selected since this concentration produces considerably less destruction of the filter paper discs as compared with 1.0 N and hydrolyzes the RNA in a shorter time period than 0.25 N.

Both sets of discs prepared with DNA were treated with 0.5 N NaOH for 90 min. to determine the percentage loss of DNA. This is necessary since the analysis for DNA is done subsequently to the RNA hydrolysis procedure when analyzing cells possessing both labeled DNA and labeled RNA. This procedure revealed a 9.1% loss of DNA prepared by the pulse-label method, and 11.9% loss of the isolated DNA. Therefore, a correction consisting of 10.5% of the total DNA radioactivity (as determined by hot TCA hydrolysis) must be subtracted from the total radioactivity lost by 0.5 N NaOH hydrolysis in order to obtain the true value for RNA radioactivity.

Following the NaOH hydrolysis procedure, the DNA discs were removed from the scintillation vials, washed in acetone, and dried. The

discs were then treated with 5% TCA at 90°C for 15, 30 and 45 min. The results are shown in Table 32. Within 30 min., 96% of the pulse-labeled DNA and 99% of the isolated DNA had been hydrolyzed. Therefore, the true value for DNA radioactivity is obtained by multiplying the radioactivity hydrolyzed by hot TCA by 1.15 (to account for that lost during NaOH hydrolysis, and that portion of DNA not hydrolyzed by hot TCA).

IX. The development and characterization of a primaquine-resistant strain of T. pyriformis:

A. Introduction: Elucidation of the biochemical/physiological changes which occur with the development of resistance to primaquine can be investigated by comparative studies with our primaquine-resistant and normal strains of T. pyriformis.

B. Methods and Results: The primaquine-resistant strain of T. pyriformis has been developed by exposing the organism to successively higher concentrations of the drug. Originally, the organism in standard growth medium (2% proteose-peptone-0.1% liver extract) was incubated with 60 µg/ml of the drug (half the lethal dose). After 4 transfers of the organism in this growth medium at 3 day intervals, the drug concentration was increased by 60 µg/ml. This procedure of increasing the drug concentration every fourth transfer was continued until a level of 2 mg/ml (4×10^{-3}M) was obtained. This is approximately 20 times the lethal dose to normal cells. Subsequently the primaquine-resistant strain has been maintained by serial transfers at 3-4 day intervals into fresh medium containing this drug concentration.

Figure 27 illustrates the growth curve for the primaquine-resistant strain of T. pyriformis. The growth of the strain is characterized by a generation time of 7½ hours, and a maximum stationary population of 64,000 cells/ml. These values compare to a generation time of 2½ hours and a maximum stationary population of 1.5 million cells/ml for normal cells.

X. Evaluation of the physiological differences between normal and primaquine resistant strains of T. pyriformis:

A. Materials and Methods:

1. Growth and maintenance of primaquine-resistant strain: The organisms were maintained in upright cotton-stoppered test tubes by transfer at 3 day intervals into fresh PPL medium containing 2 mg/ml primaquine. For experimental purposes, the organisms were grown in this medium in 500 ml Erlenmeyer flasks containing 100 ml of medium or in 2,250 ml Fernbach culture flasks containing 1000 ml of medium. Harvesting and washing of the cells were done as for the normal strain.

2. Evaluation of respiration of the primaquine-resistant strain: The respiratory rate of the resistant cells were compared to that of normal cells by the use of a Yellow Springs Instrument Co., Model 53, Biological Oxygen Monitor. Two reaction cells were used, one containing 2.0 ml of the normal cells and the other containing 2.0 ml of the resistant cells. The cells were suspended in Ryley's buffer at approximately 40,000 cells/ml. The air-saturated suspensions were allowed to equilibrate to 29°C, and the respiration was followed for approximately 20 min. The oxygen consumption for both strains were determined in the presence and absence of primaquine.

B. Results: Our initial results on respiratory rate indicate that primaquine resistant cells utilize oxygen at a much slower rate than normal cells. This, however, may be due to the slower growth rate of the resistant cells (generation time 7½ hrs. as compared to 2½ hrs. for normal cells). Preliminary results indicate that the resistant cells gradually regain their normal respiratory rate when placed in primaquine-free growth medium.

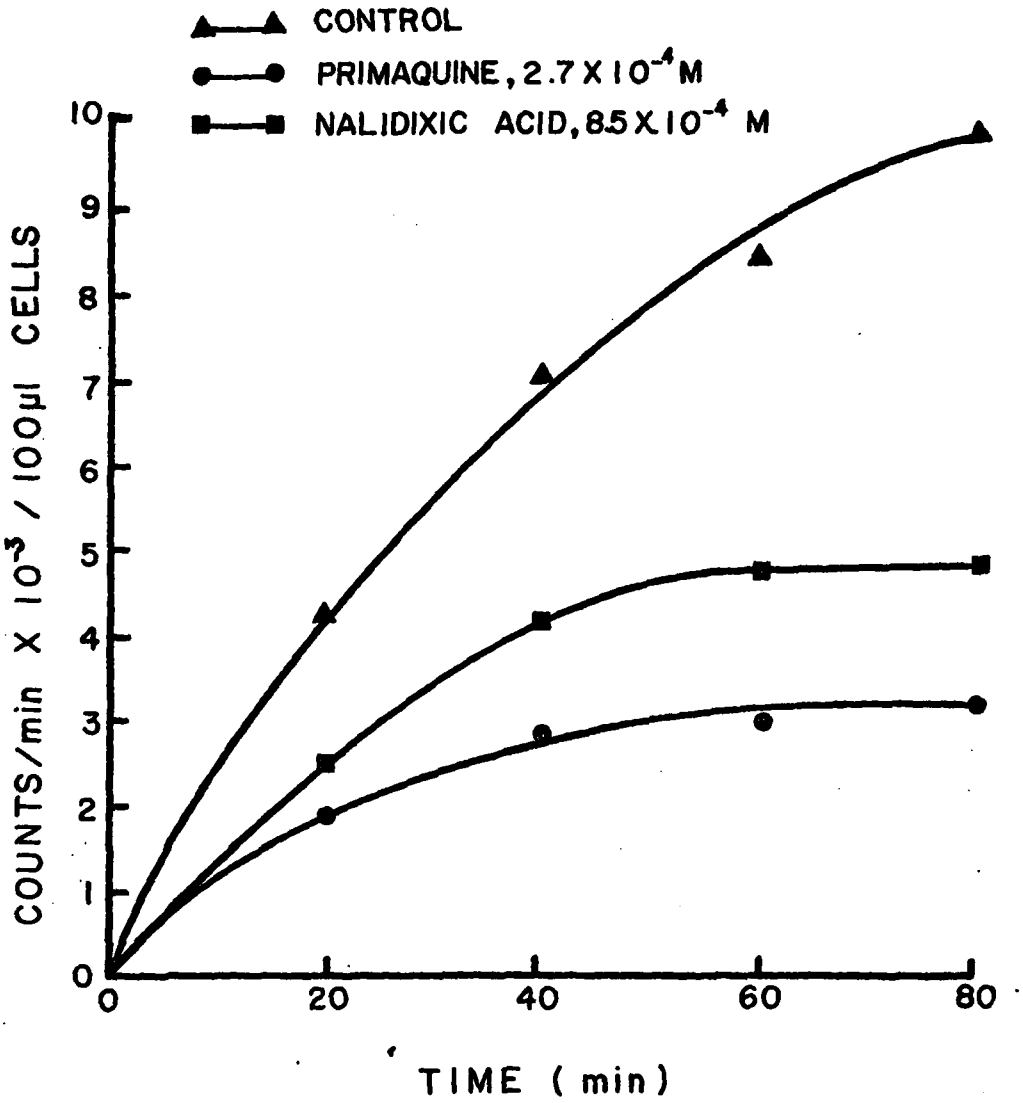


Figure 1. The effects of primaquine and nalidixic acid on incorporation of ^{14}C-thymidine. The drugs and radioactive precursor were added at EHT and 0.1 ml samples were removed at 0, 20, 40, 60, and 80 min. and processed by the filter paper disc procedure to determine incorporation. The cell population was 100,000/ml.

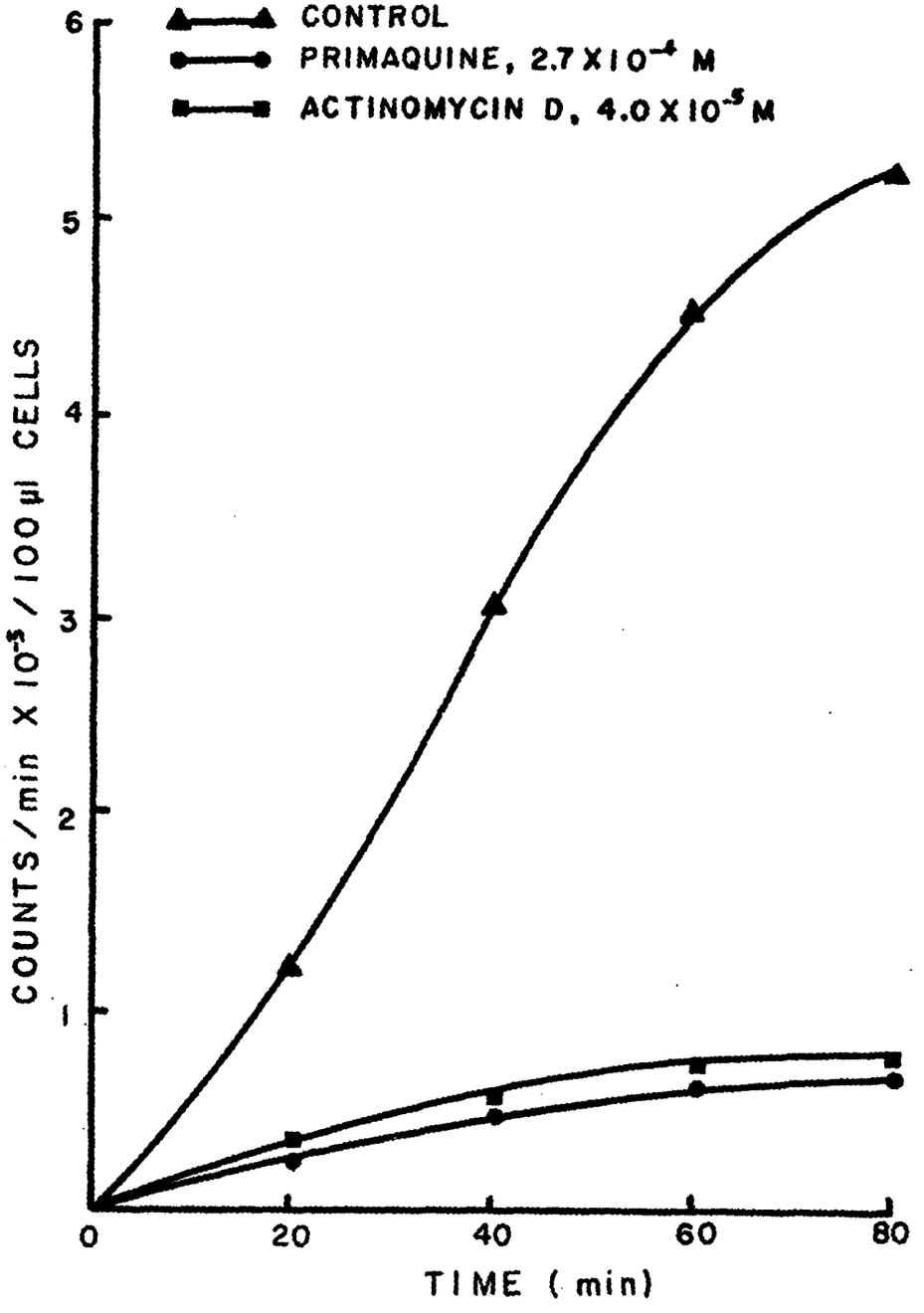


Figure 2. The effects of primaquine and actinomycin D on incorporation of ^{14}C-uridine. The conditions are as described in Figure 1.

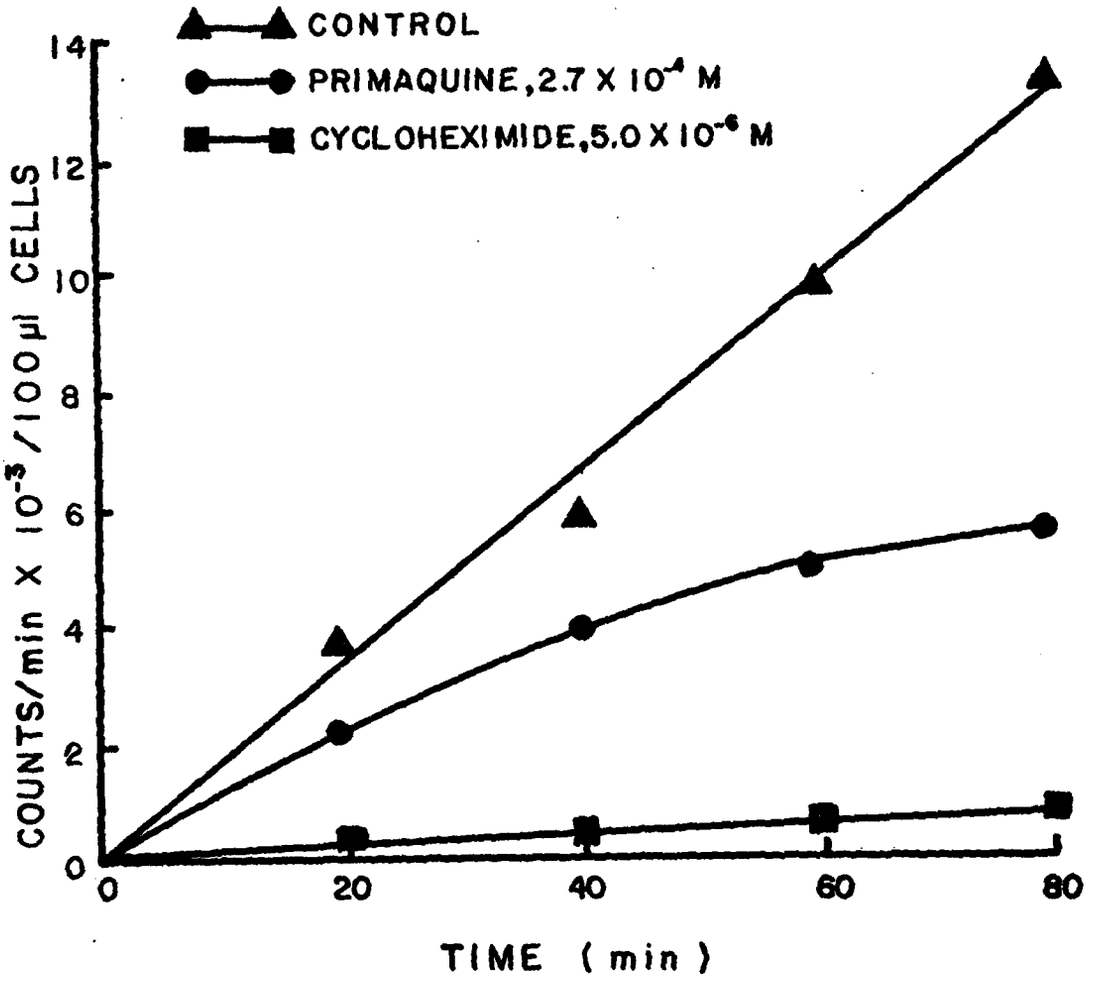


Figure 3. The effects of primaquine and cycloheximide on incorporation of ^{14}C-amino acids. The conditions are as described in Figure 1, except the cell population was 130,000/ml.

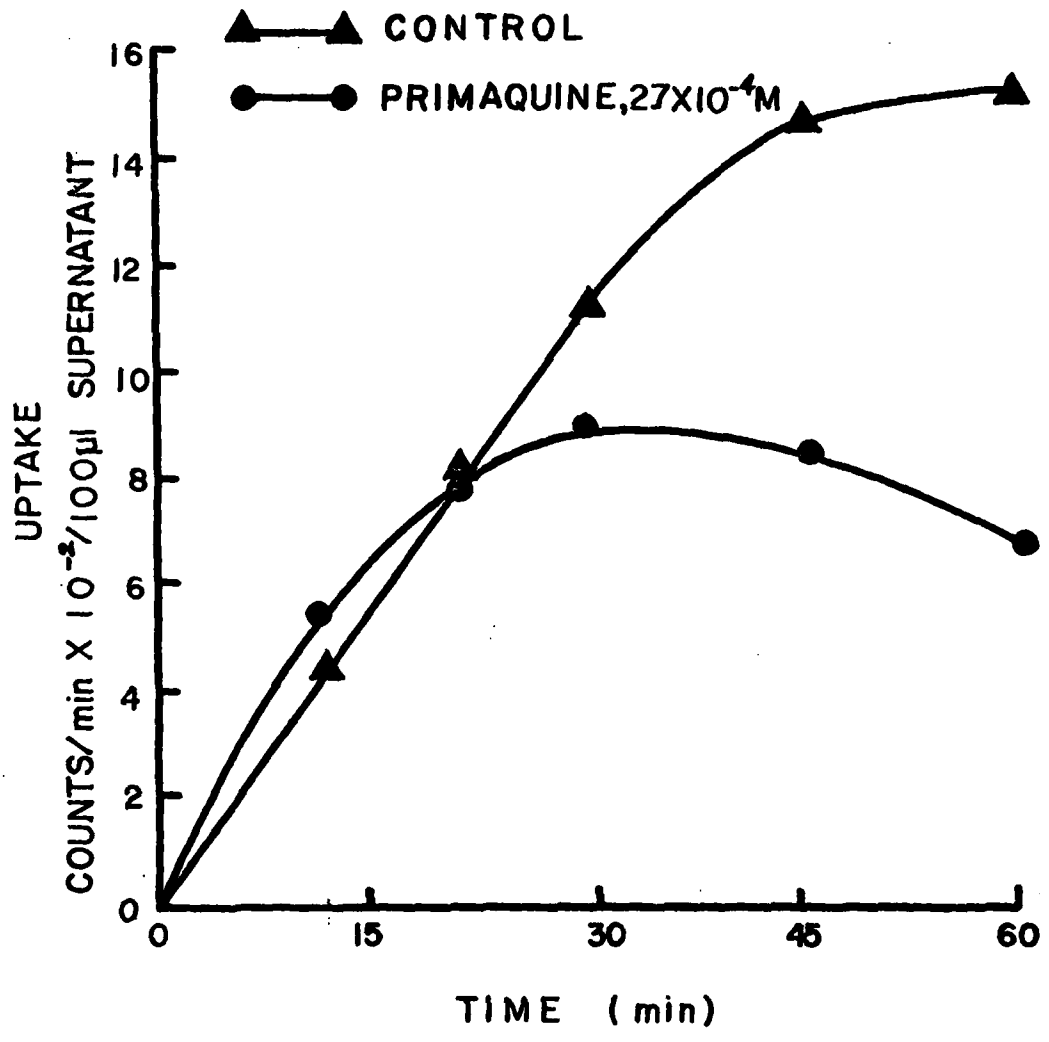


Figure 4. The effects of primaquine on uptake of ^{14}C-thymidine by cells in which DNA synthesis is blocked by nalidixic acid. At EHT, nalidixic acid (final concentration, 8.5 X 10^{-4} M) was added and the cells were incubated for 20 min. before adding primaquine and the radioactive precursor. To determine uptake, samples were removed at 20, 30, 40, 50, 65, and 80 min. after EHT (time zero = EHT plus 20 min.), the cells sedimented, and the radioactivity of 0.1 ml samples of the supernatants determined after being placed on filter paper discs and dried. The cell population was 105,000/ml.

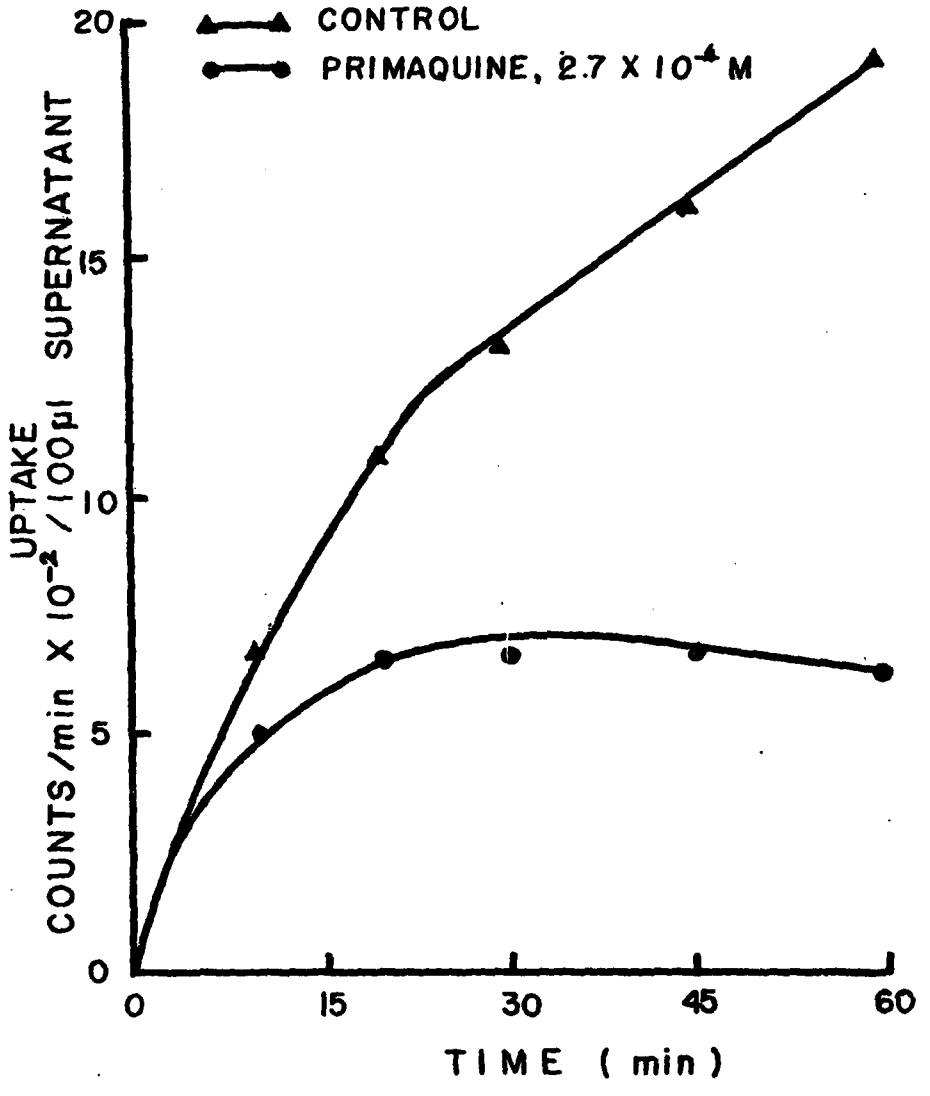


Figure 5. The effects of primaquine on uptake of ^{14}C-uridine by cells in which RNA synthesis is blocked by actinomycin D. At EHT, actinomycin D (final concentration, 4.0×10^{-5} M) was added and the cells were incubated for 20 min. before adding the drug and radioactive precursor. Other conditions are as described in Figure 4, except the cell population was 106,000/ml.

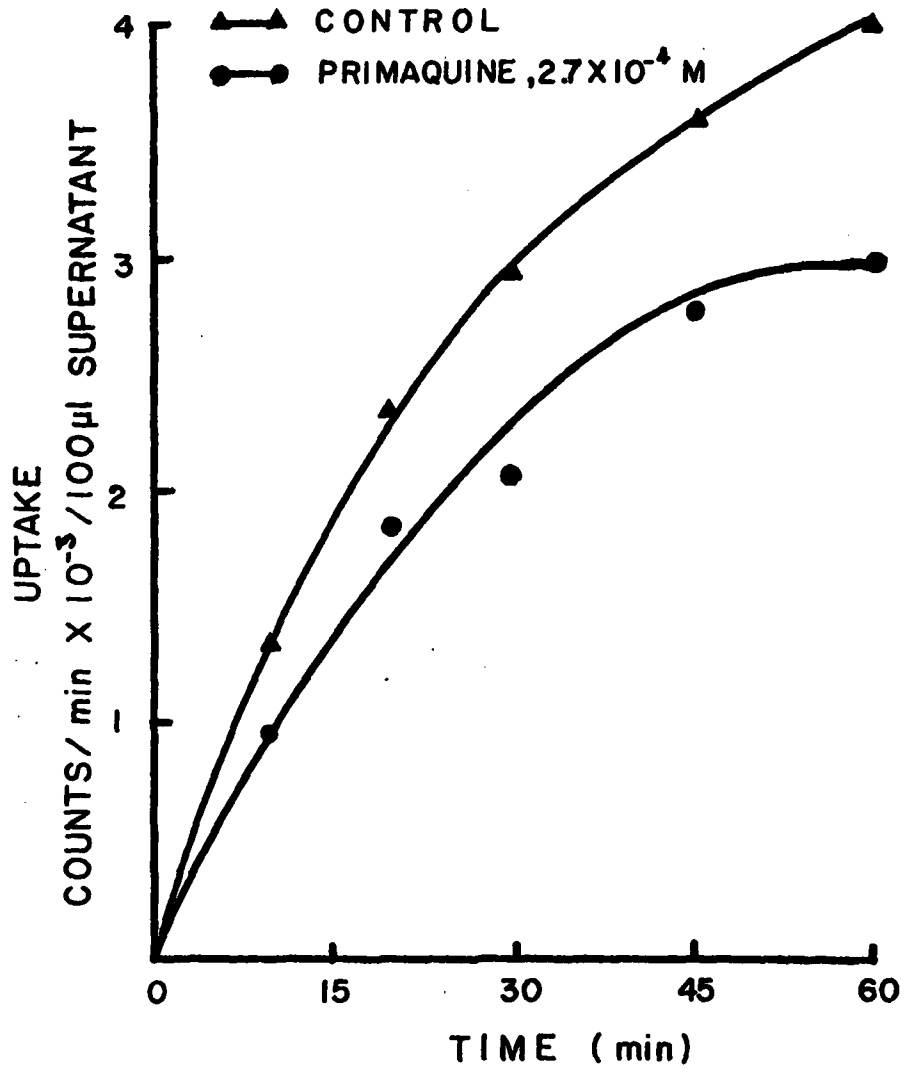


Figure 6. The effects of primaquine on uptake of ^{14}C-amino acids by cells in which protein synthesis is blocked by cycloheximide. At EHT, cycloheximide (final concentration, 5.0×10^{-6} M) was added and the cells were incubated for 20 min. before adding the drug and radioactive precursor. Other conditions are as described in Figure 4, except the cell population was 193,000/ml.

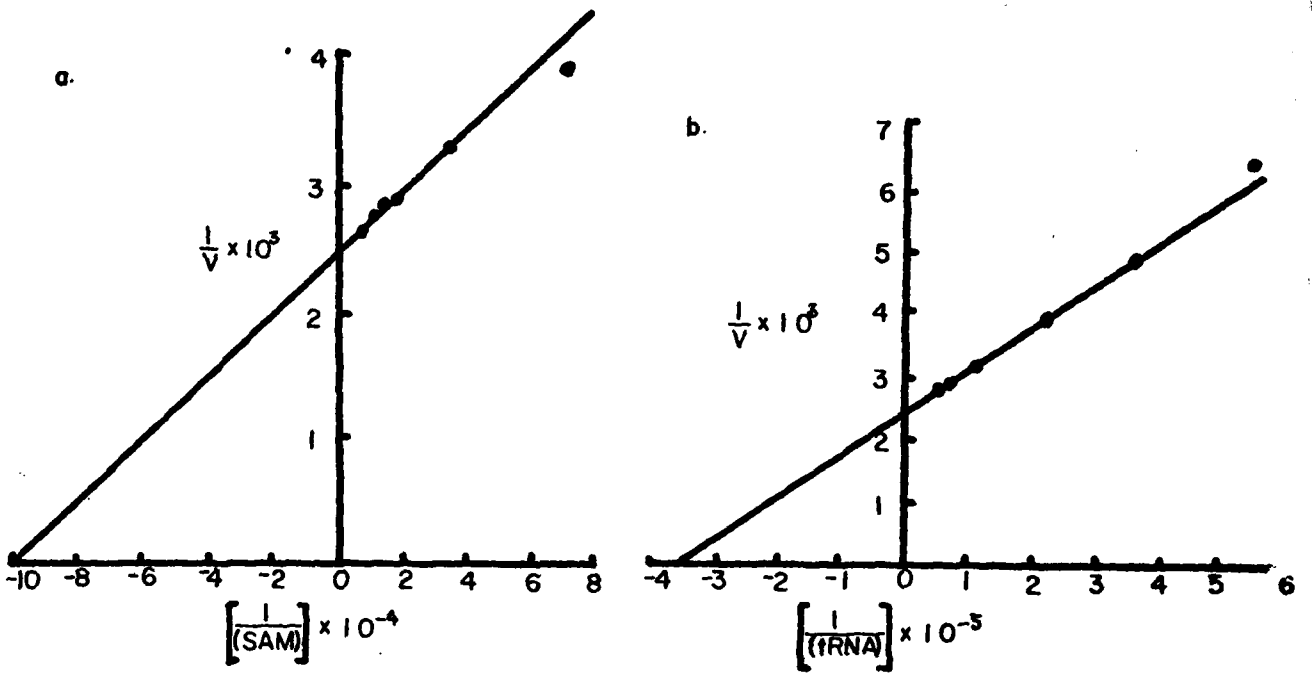


Figure 7. Reciprocal plots for the methylation of transfer RNA with varying concentrations of SAM (a) and tRNA (b) by tRNA methylases of Tetrahymena pyriformis. V=^{14}C-methyl incorporated into tRNA (picomoles/hr); (tRNA)=concentration of tRNA based on an M.W. of 27,000; (SAM)=concentration of ^{14}C-methyl-S-adenosylmethionine.

(a) K_m(SAM)=1.0 X 10^{-5} M. (b) K_m(tRNA)=2.9 X 10^{-6} M.

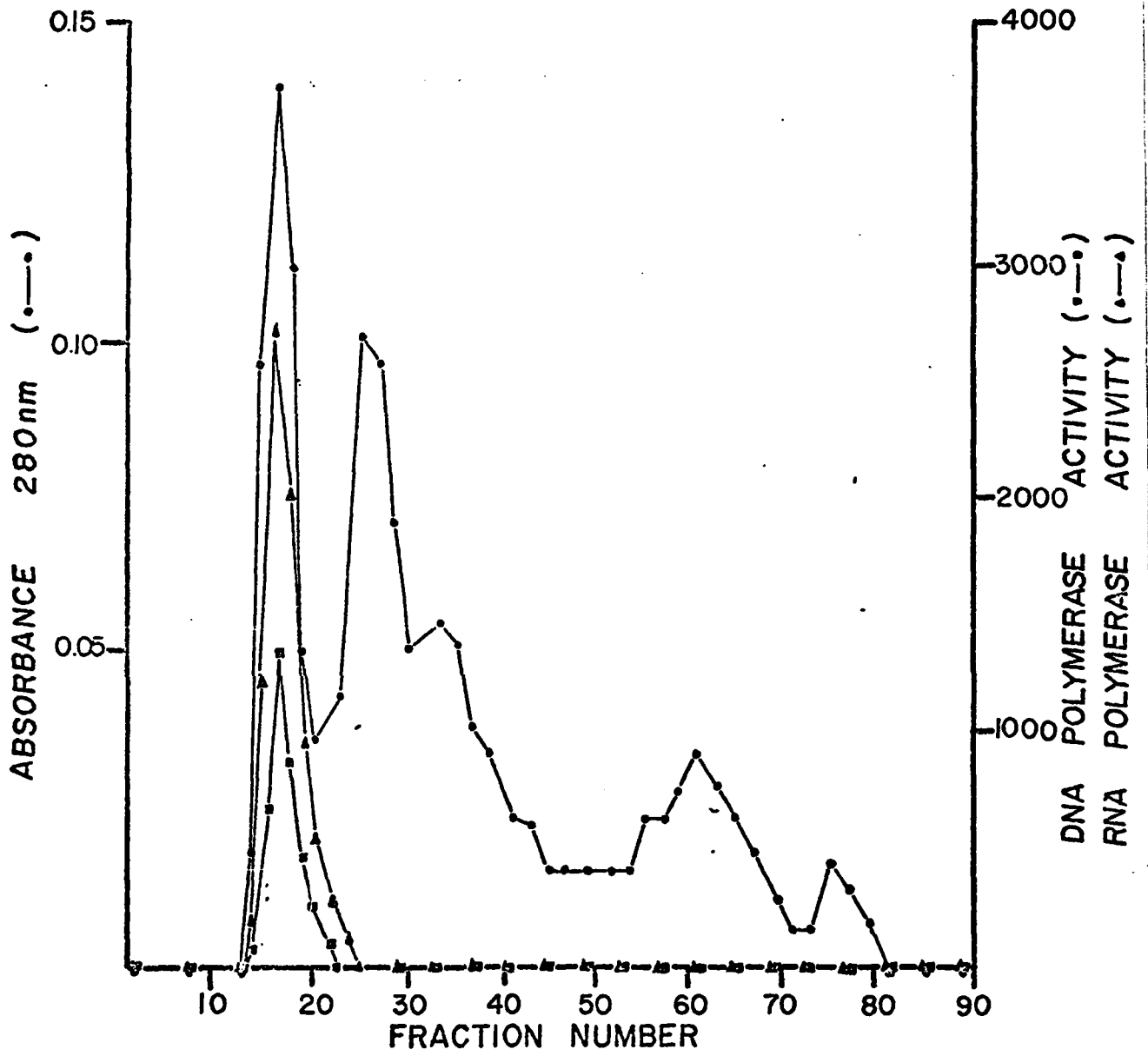


Figure 8. Sephadex G-100 elution pattern of DNA and RNA polymerases.

The 105,000 X g supernatant from lysed nuclei was chromatographed on a column of Sephadex G-100, 2 X 35 cm, which was equilibrated with TMG buffer. The enzyme was eluted in fractions of 3.0 ml with TMG buffer and 100 µl aliquots were assays for DNA and RNA polymerase activity as described in the text. Activity is defined as counts per minute incorporated in the 60 min assay.

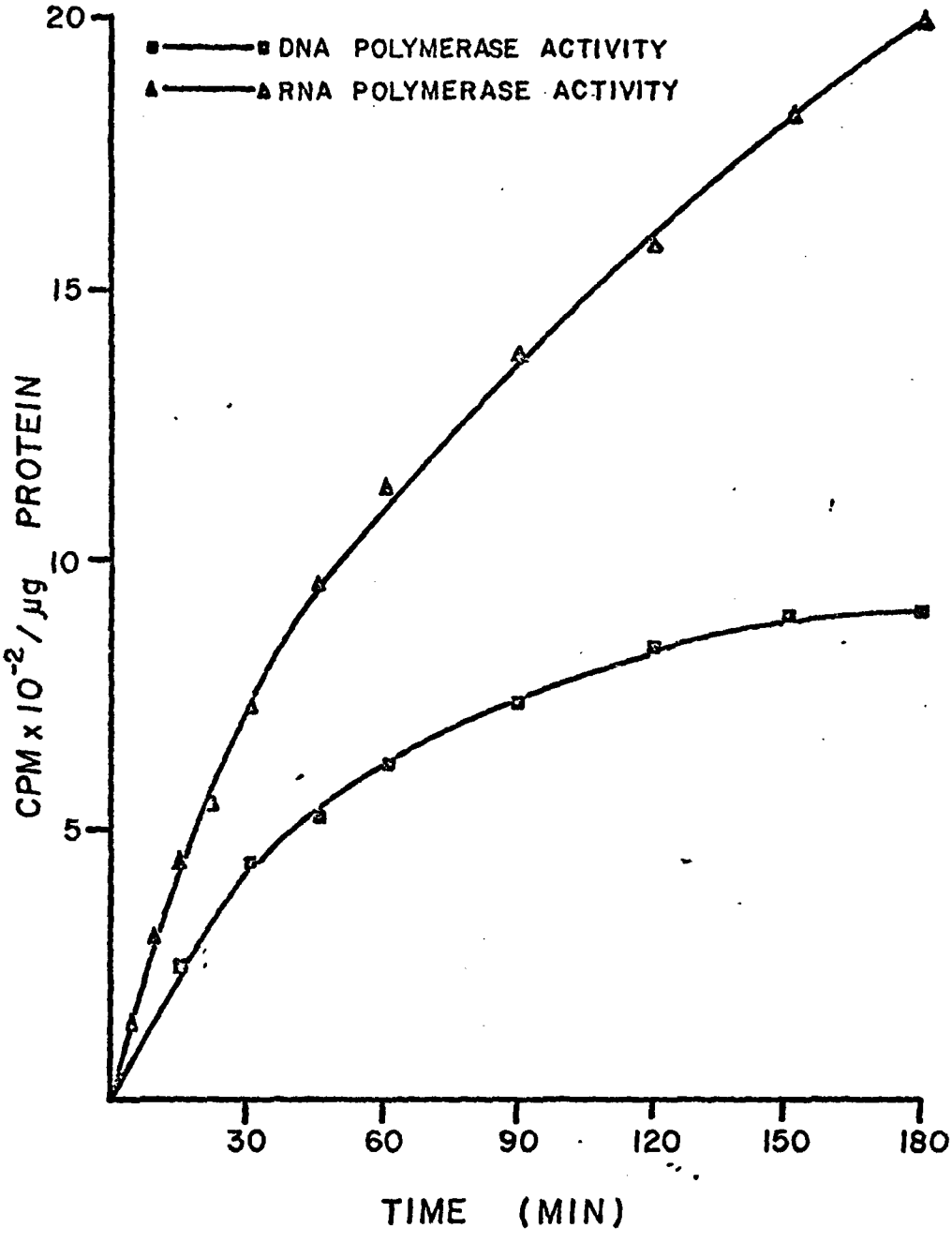


Figure 9. Time course of the DNA and RNA polymerase reactions by solubilized polymerases.

Assay conditions are described in the text, with samples being assayed at the indicated times.

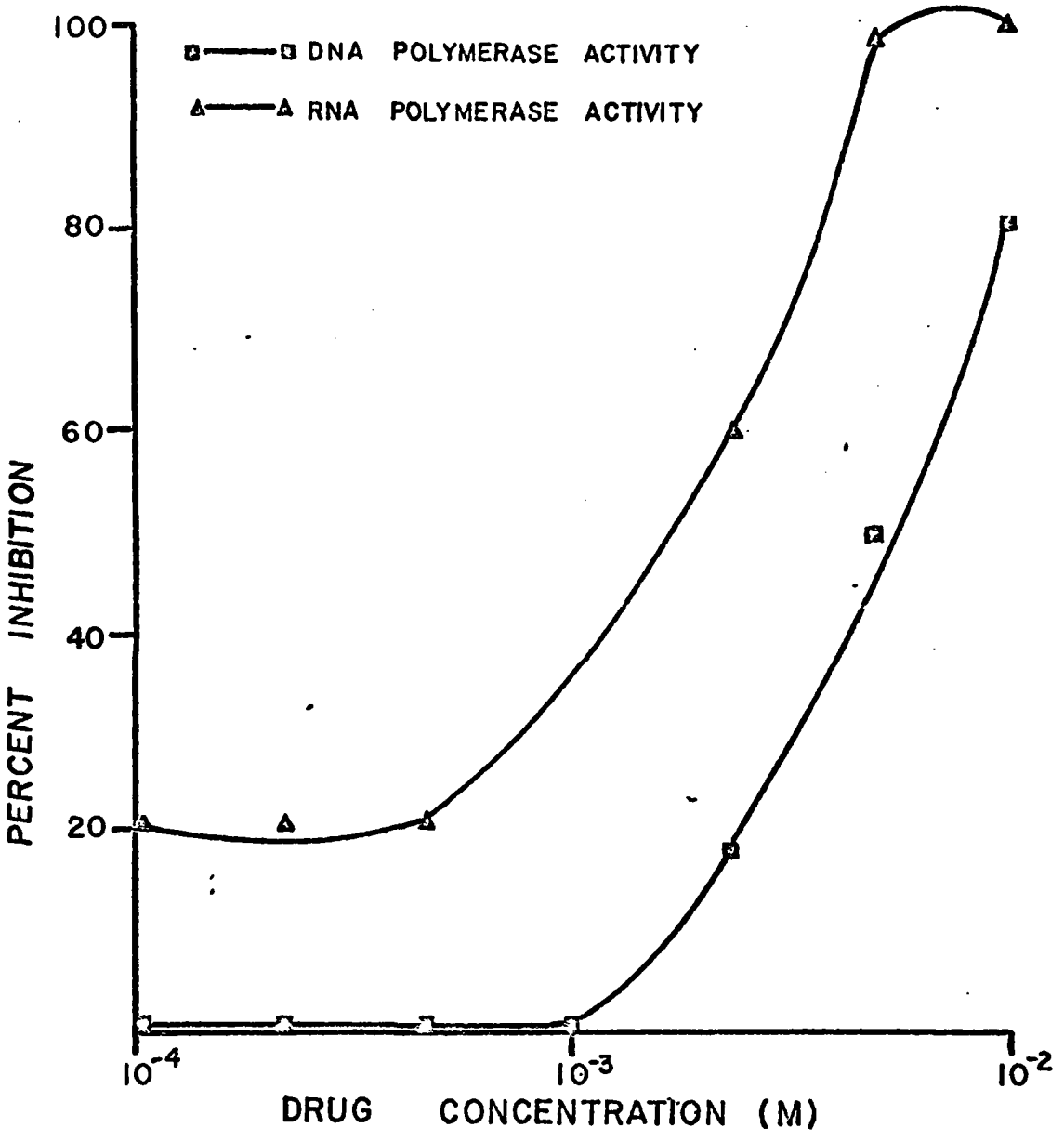


Figure 10. Dose-response for inhibition of DNA and RNA syntheses with solubilized polymerases by primaquine.

Assay conditions are as described in the text with primaquine being added to the complete reaction mixtures for DNA and RNA syntheses by the solubilized polymerases.

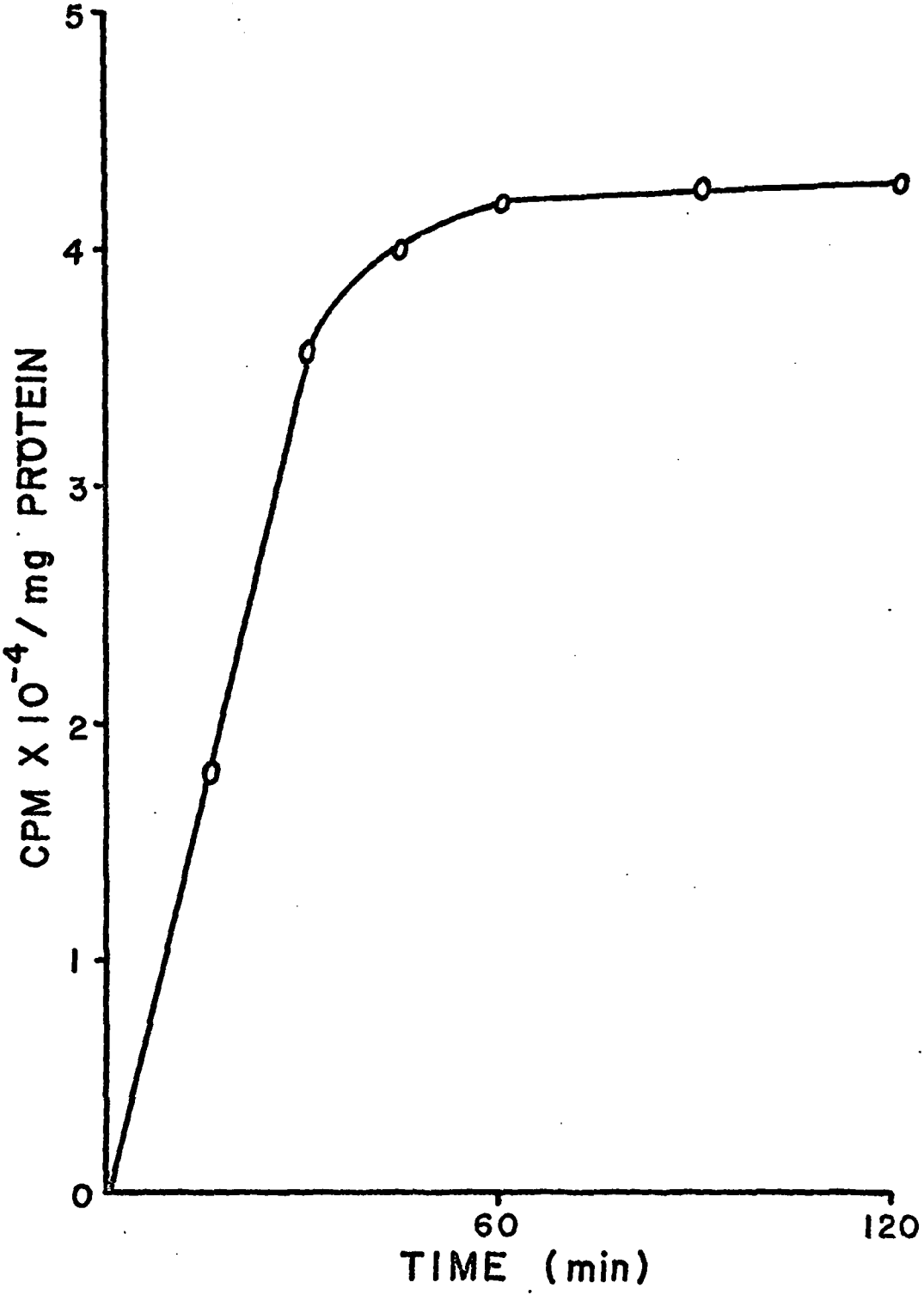


Figure 11. Time course for lipid synthesis by the crude homogenate. The reaction mixture is as described in Materials and Methods, with samples being assayed at the indicated times by the filter paper disc procedure for lipid synthesis.

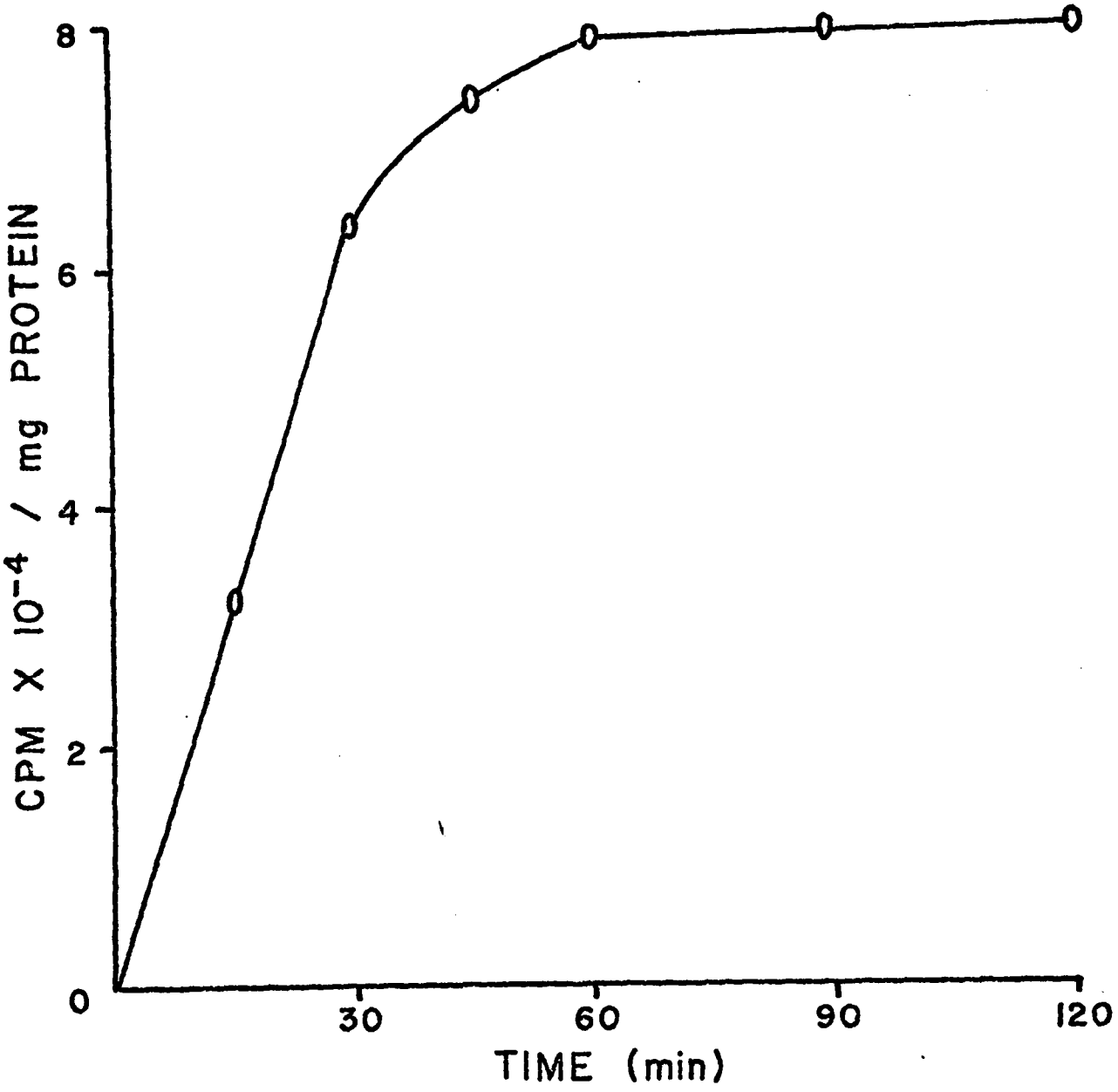


Figure 12. Time course for lipid synthesis by the mitochondrial fraction. The reaction mixture is as described in Materials and Methods, with samples being assayed at the indicated times by the filter paper disc procedure for lipid synthesis.

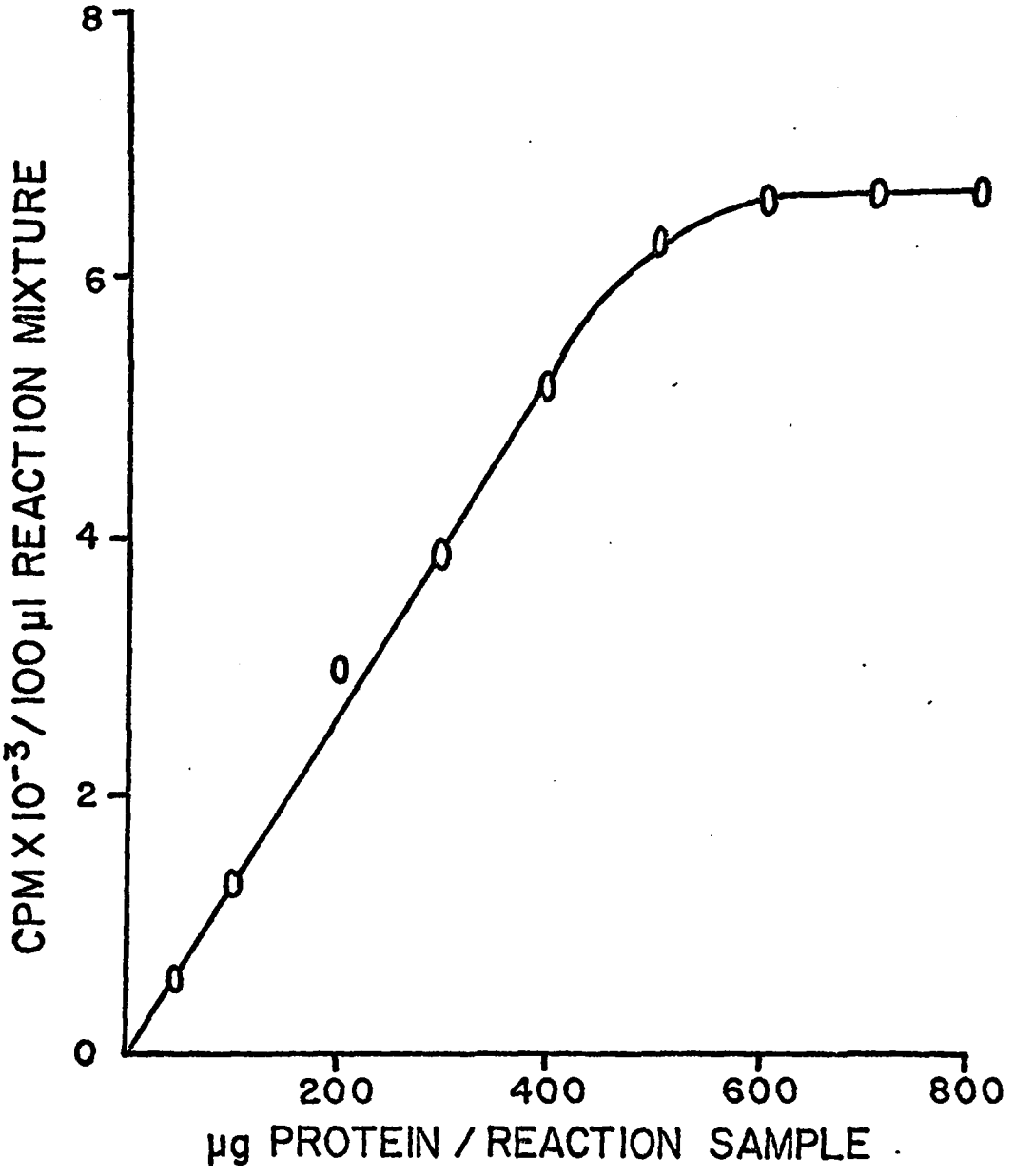


Figure 13. Protein concentration for lipid synthesis in the mitochondrial fraction. The reaction mixture is as described in Materials and Methods. Samples were taken at 0 and 30 min and assayed by the filter paper disc procedure for lipid synthesis.

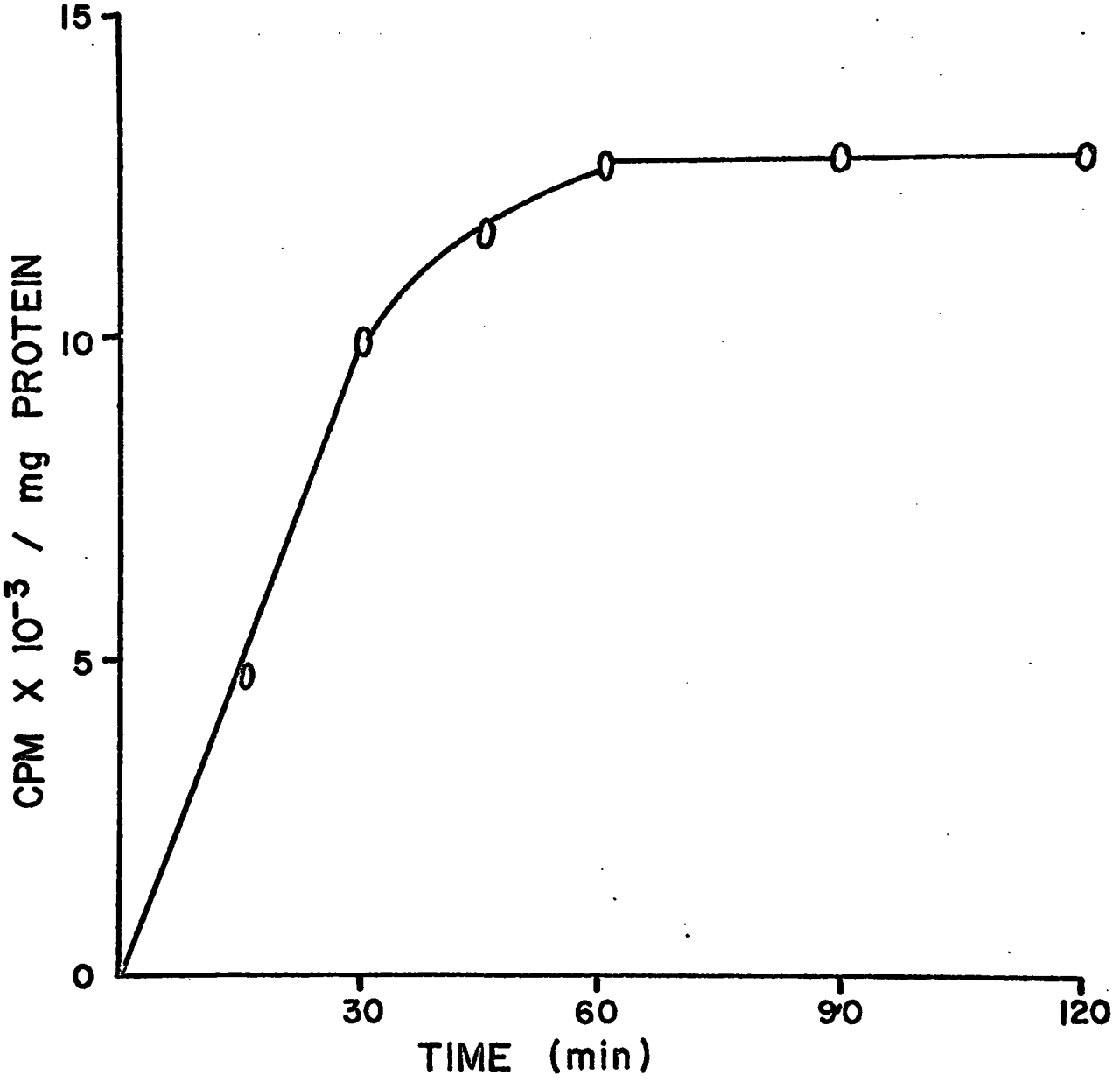


Figure 14. Time course for lipid synthesis by the microsomal fraction. The reaction mixture is as described in Materials and Methods, with samples being assayed at the indicated times by the filter paper disc procedure for lipid synthesis.

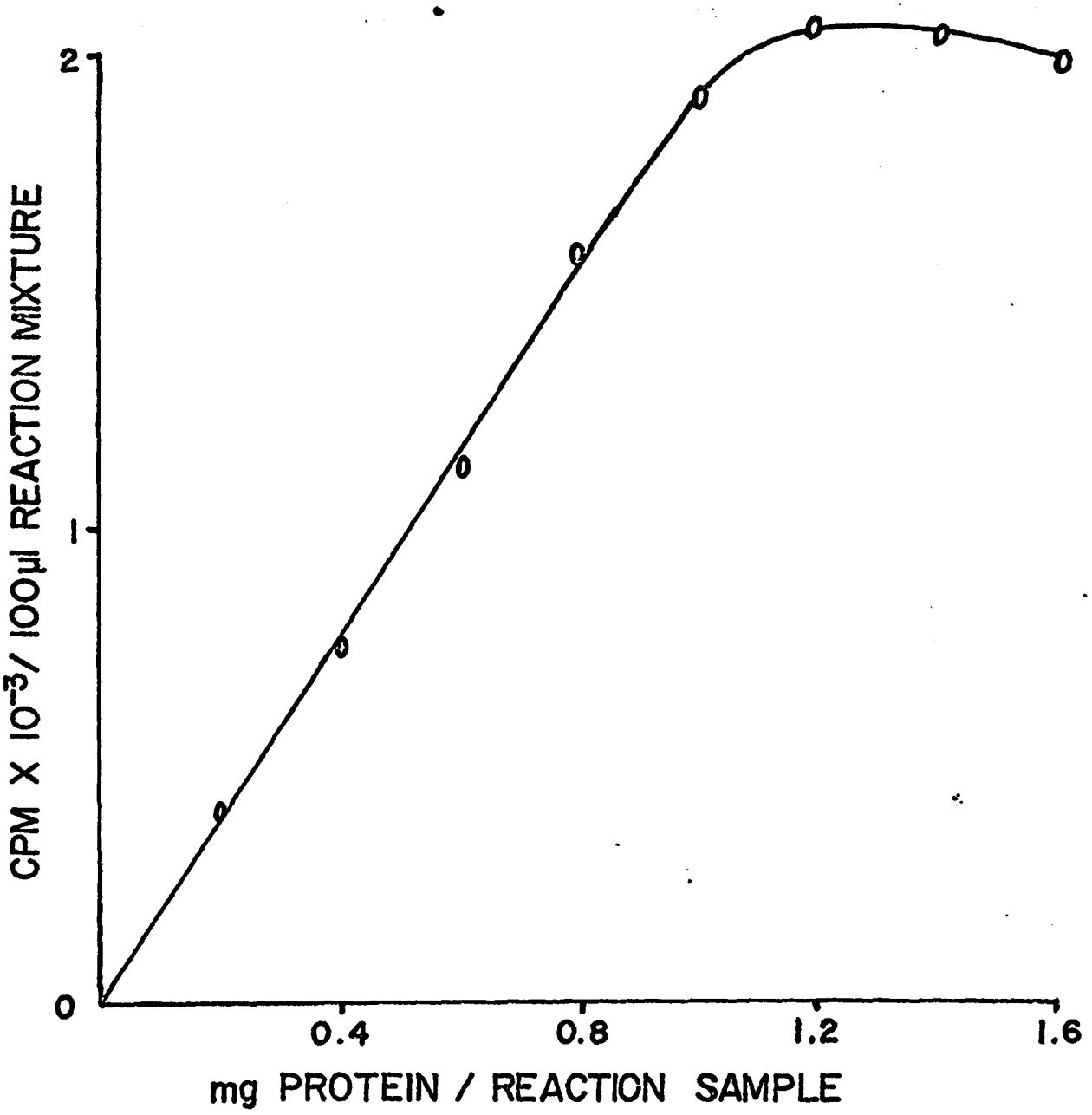


Figure 15. Protein concentration for lipid synthesis in the microsomal fraction. The reaction mixture is as described in Materials and Methods. Samples were removed at 0 and 30 min and assayed by the filter paper disc procedure for lipid synthesis.

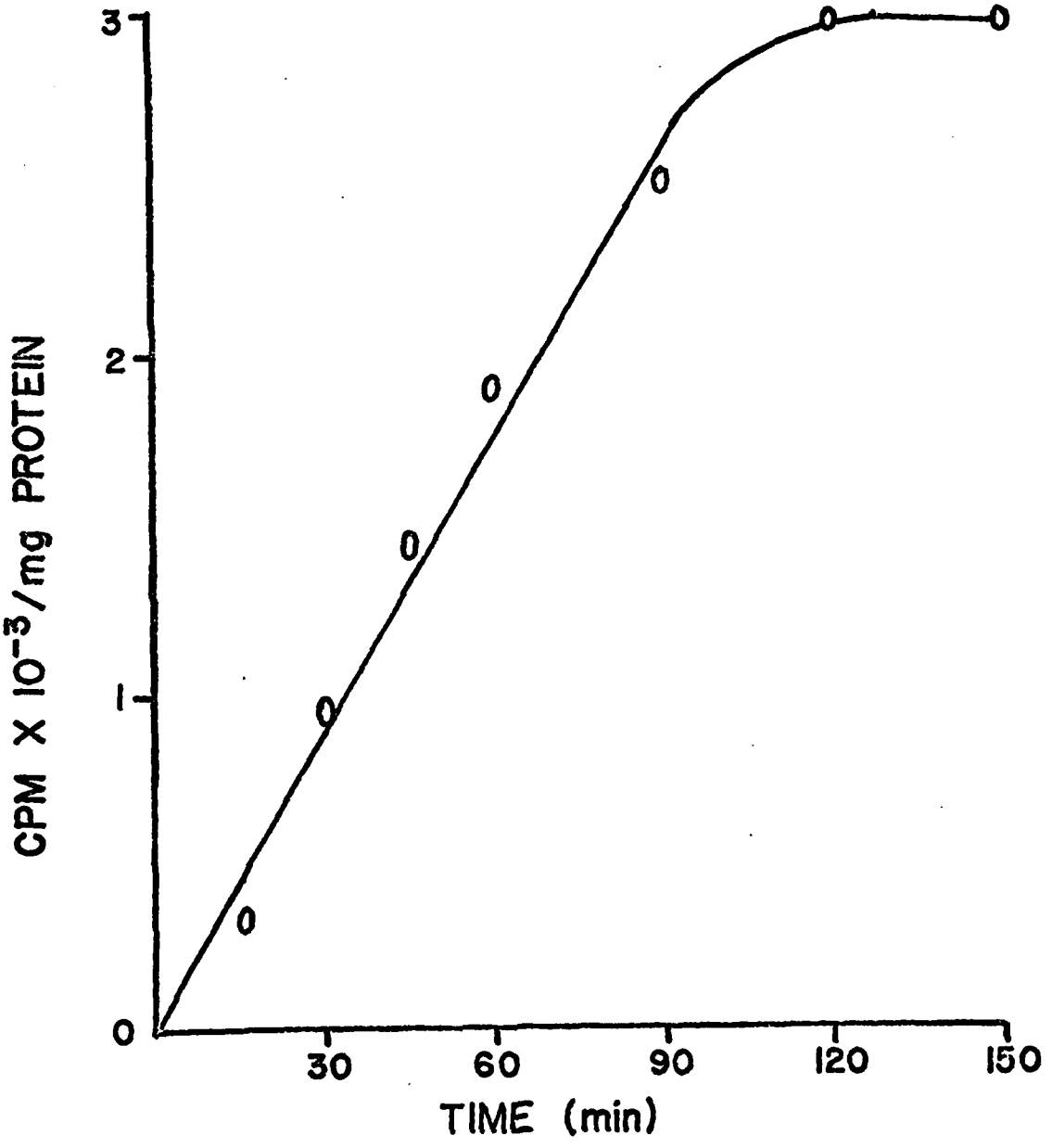


Figure 16. Time course for lipid synthesis by the soluble cell fraction. The reaction mixture is as described in Materials and Methods with samples being assayed at the indicated times by the filter paper disc procedure for lipid synthesis.

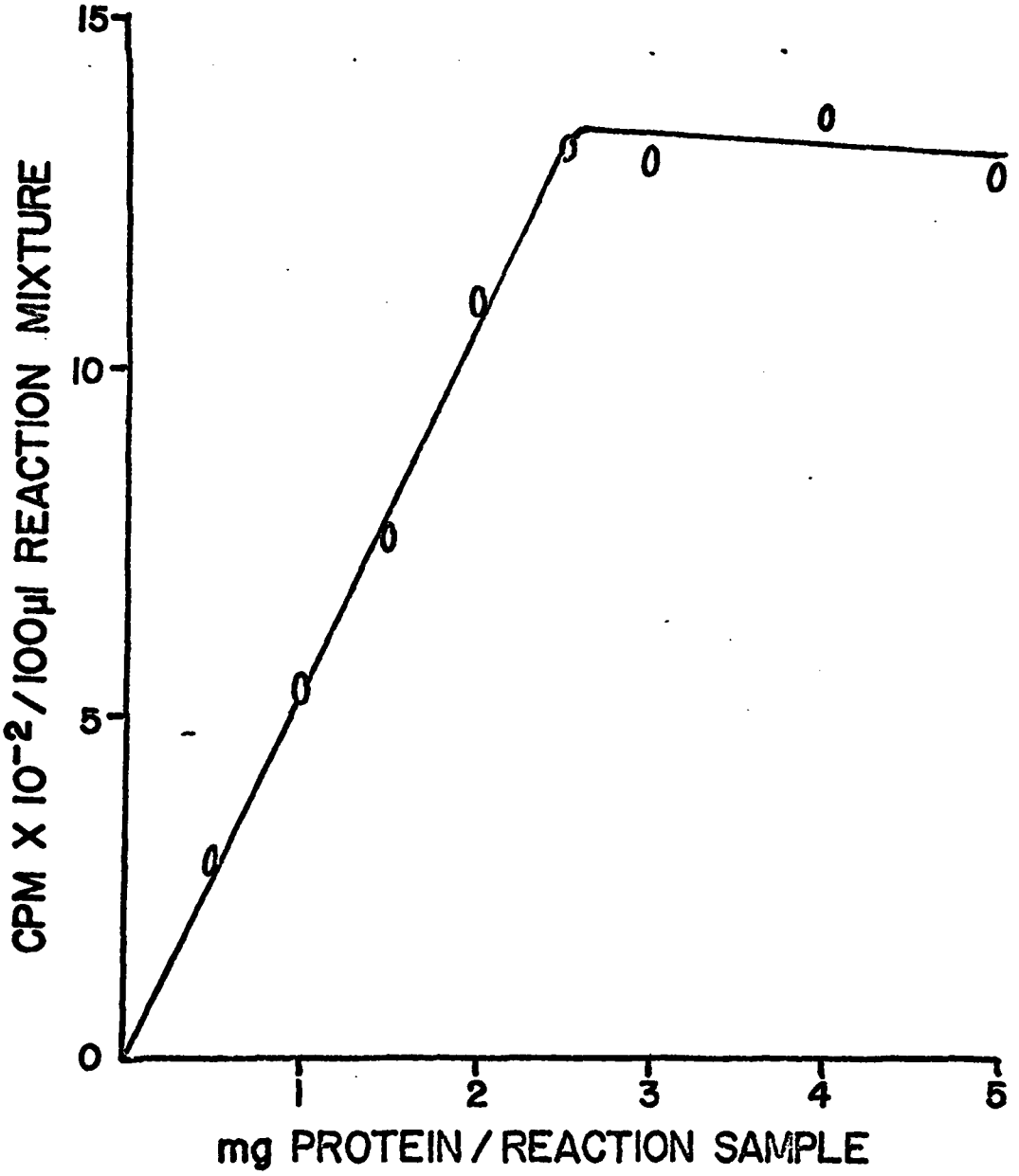


Figure 17. Protein concentration for lipid synthesis in the soluble cell fraction. The reaction mixture is as described in Materials and Methods. Samples were taken at 0 and 30 min and assayed by the filter paper disc procedure for lipid synthesis.

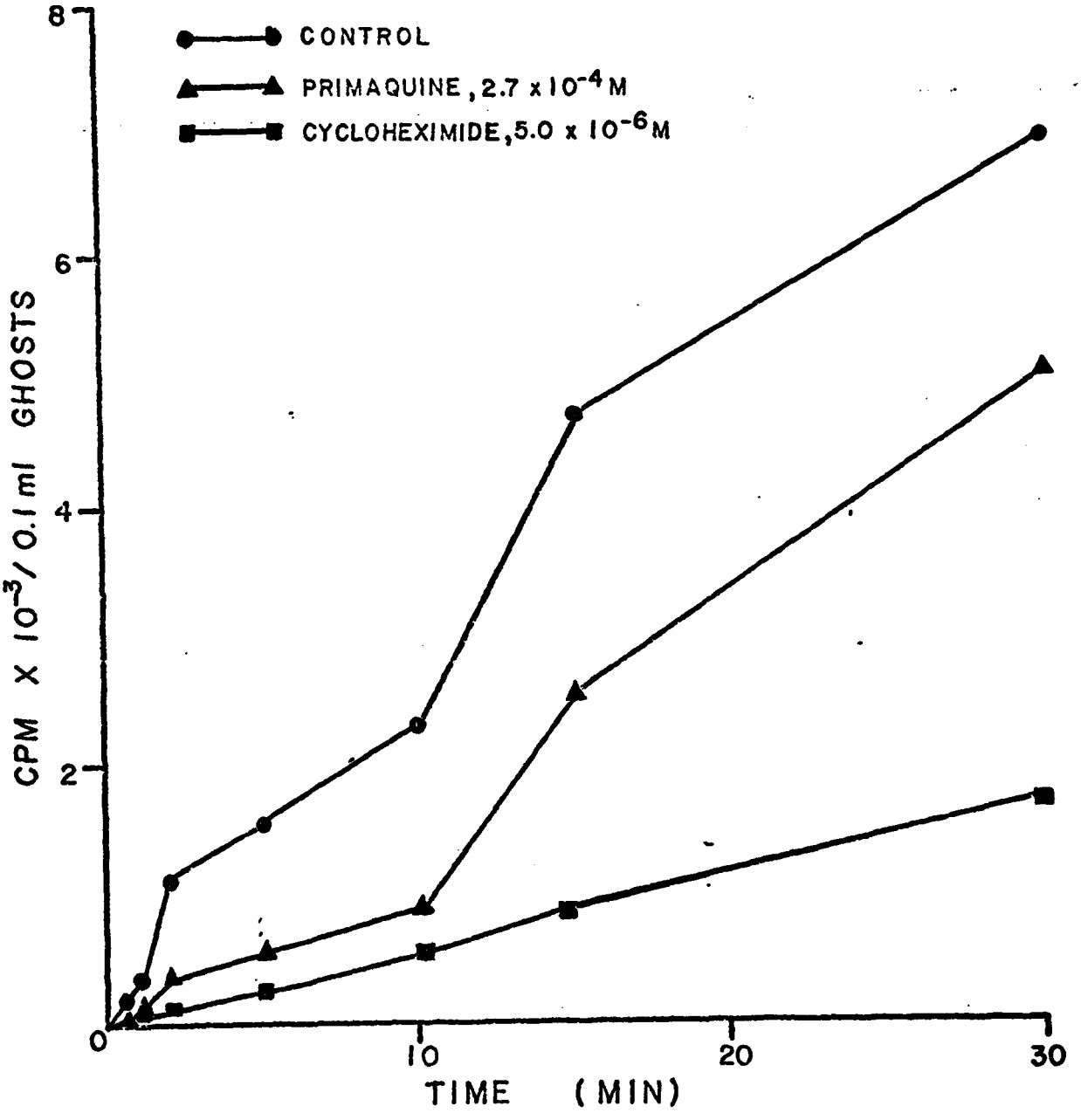


Figure 18. Binding of ^{14}C-amino acids to cell ghosts.

Cells were incubated with a ^{14}C-amino acid mixture and samples were removed at the indicated times for preparation of cell ghosts as described in the text. The CPM represent the amount of amino acids associated with the isolated ghosts.

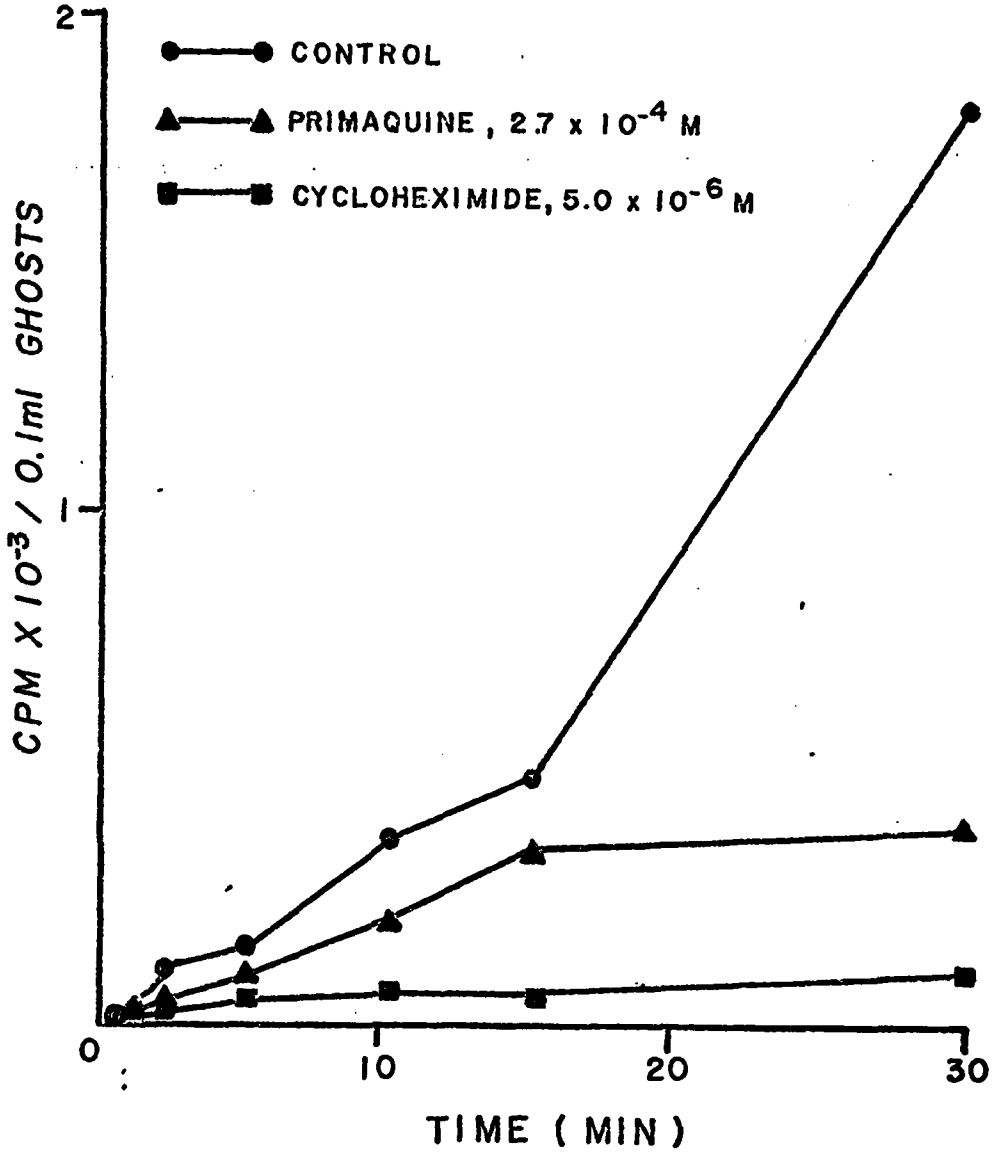


Figure 19. Incorporation of ^{14}C-amino acids into cell ghost proteins.

^{14}C-amino acids were processed by the filter paper disc procedure to measure incorporation of amino acids into cell ghost proteins. The samples represent duplicates to those of Figure 4.

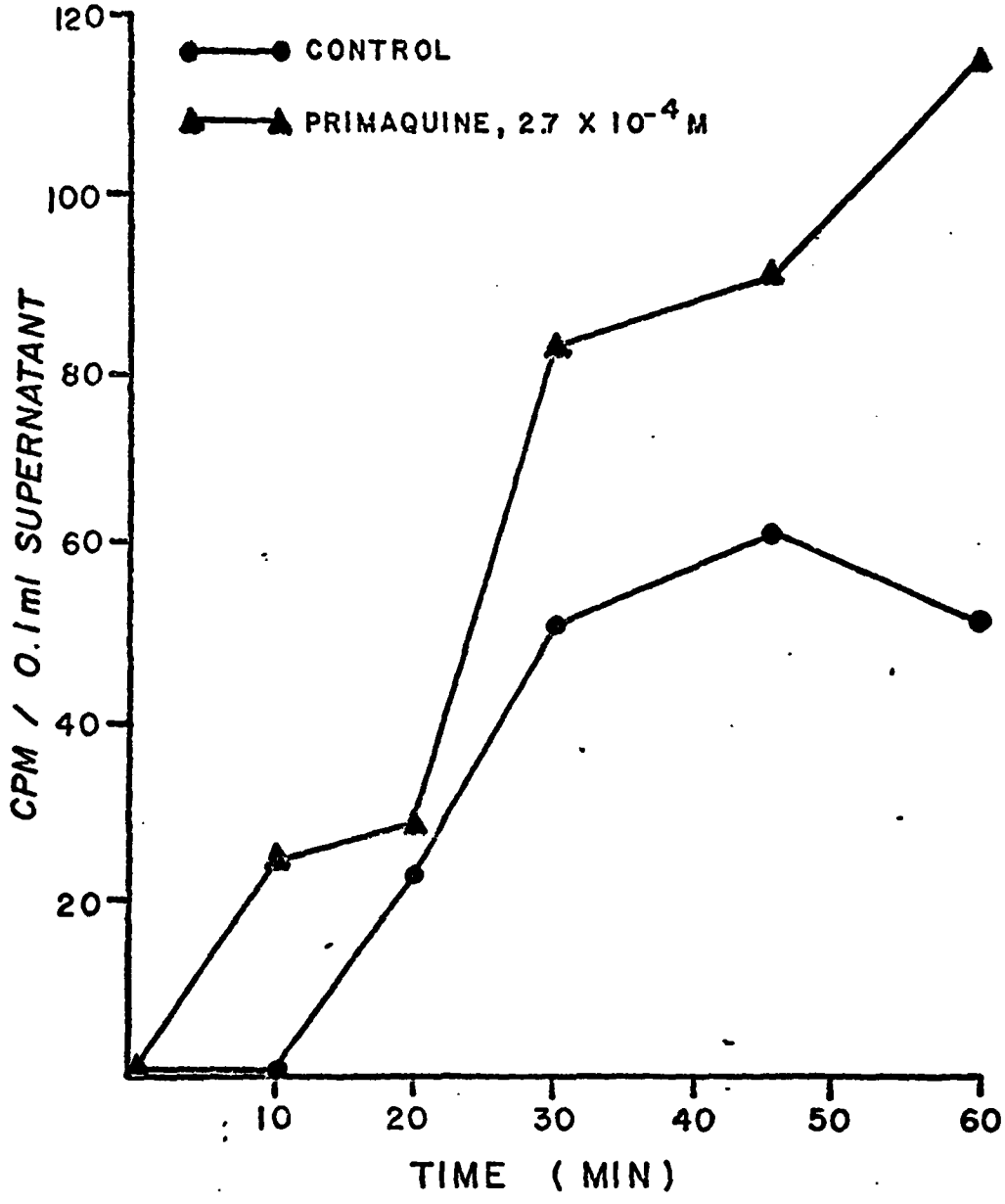


Figure 20. Release of ^{14}C-amino acids from intracellular pools in the presence and absence of primaquine.

^{14}C-amino acid mixture in the presence of cycloheximide. The cells were washed and resuspended in fresh medium containing cycloheximide. The appearance of the labeled amino acids in the supernatant represents loss from the intracellular pool.

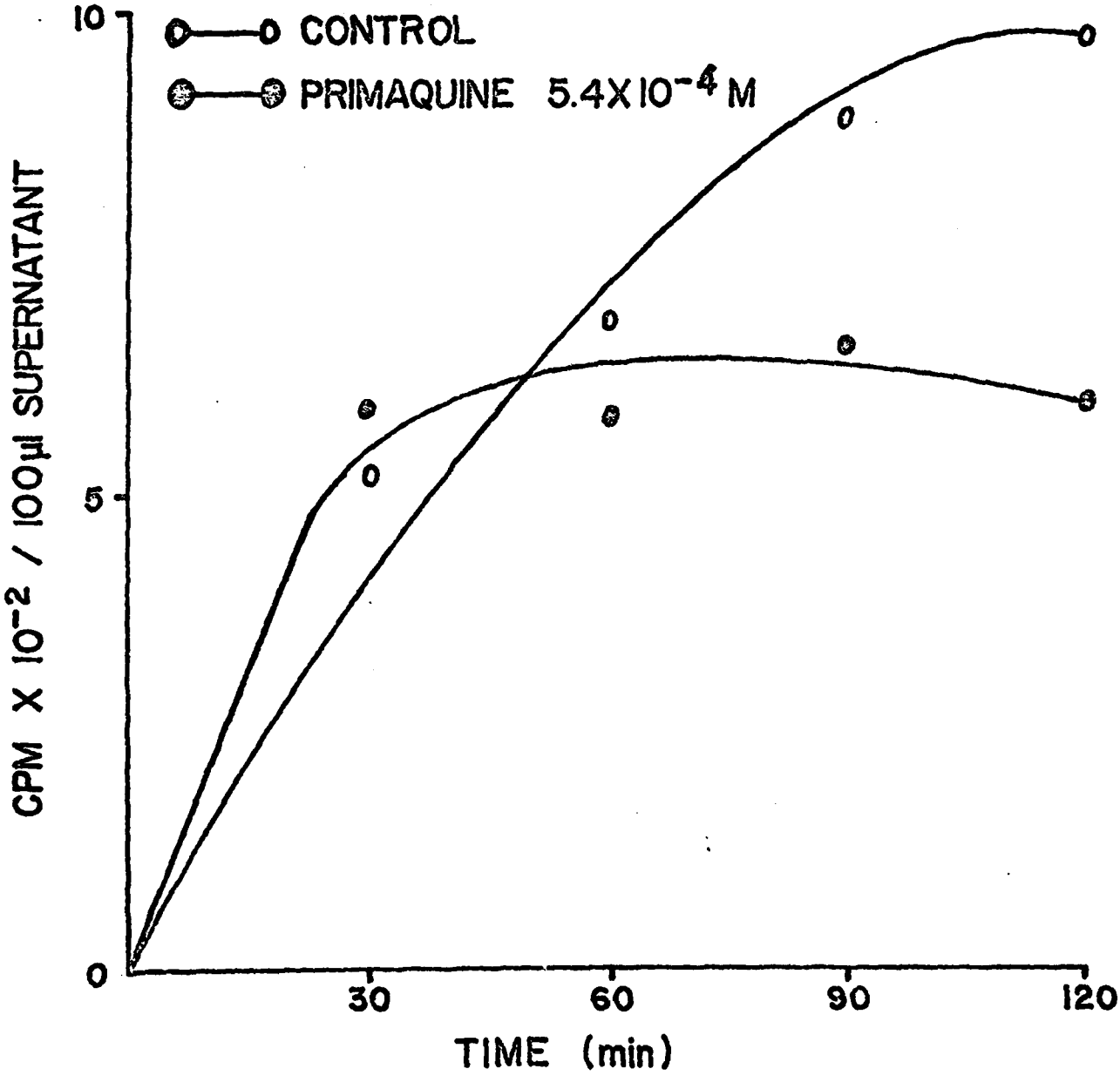


Figure 21. The effects of primaquine on uptake of ^{14}C-cycloleucine. Primaquine and the radioactive precursor were added at 0 min and samples were removed at 30, 60, 90 and 120 min, the cells were sedimented and the radioactivity of 0.1 ml samples of the supernatants was determined after being placed on filter paper discs and dried.

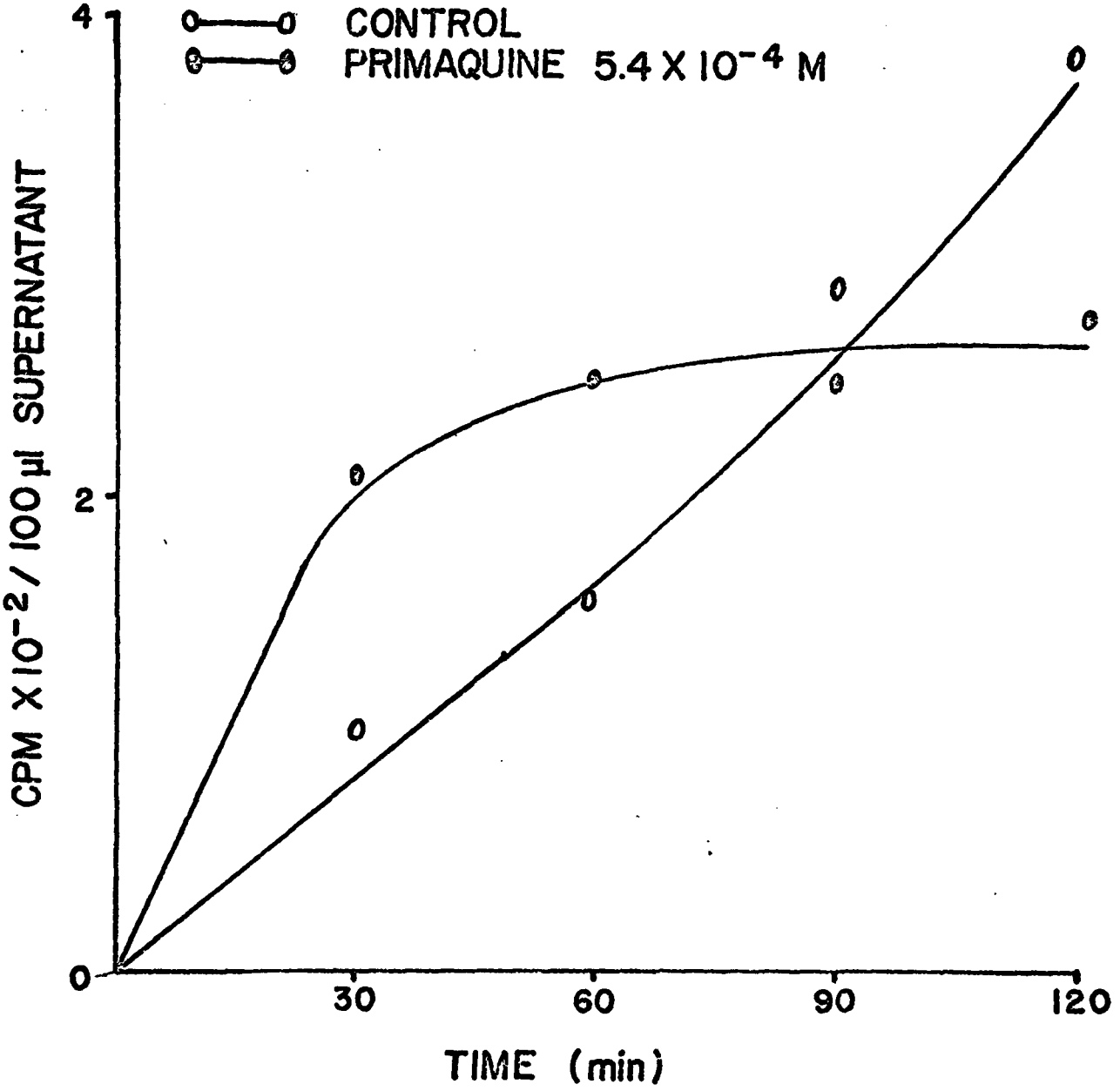


Figure 22. The effects of primaquine on uptake of ^{14}C-alpha-aminoisobutyric acid. The conditions are as described in legend to figure 8.

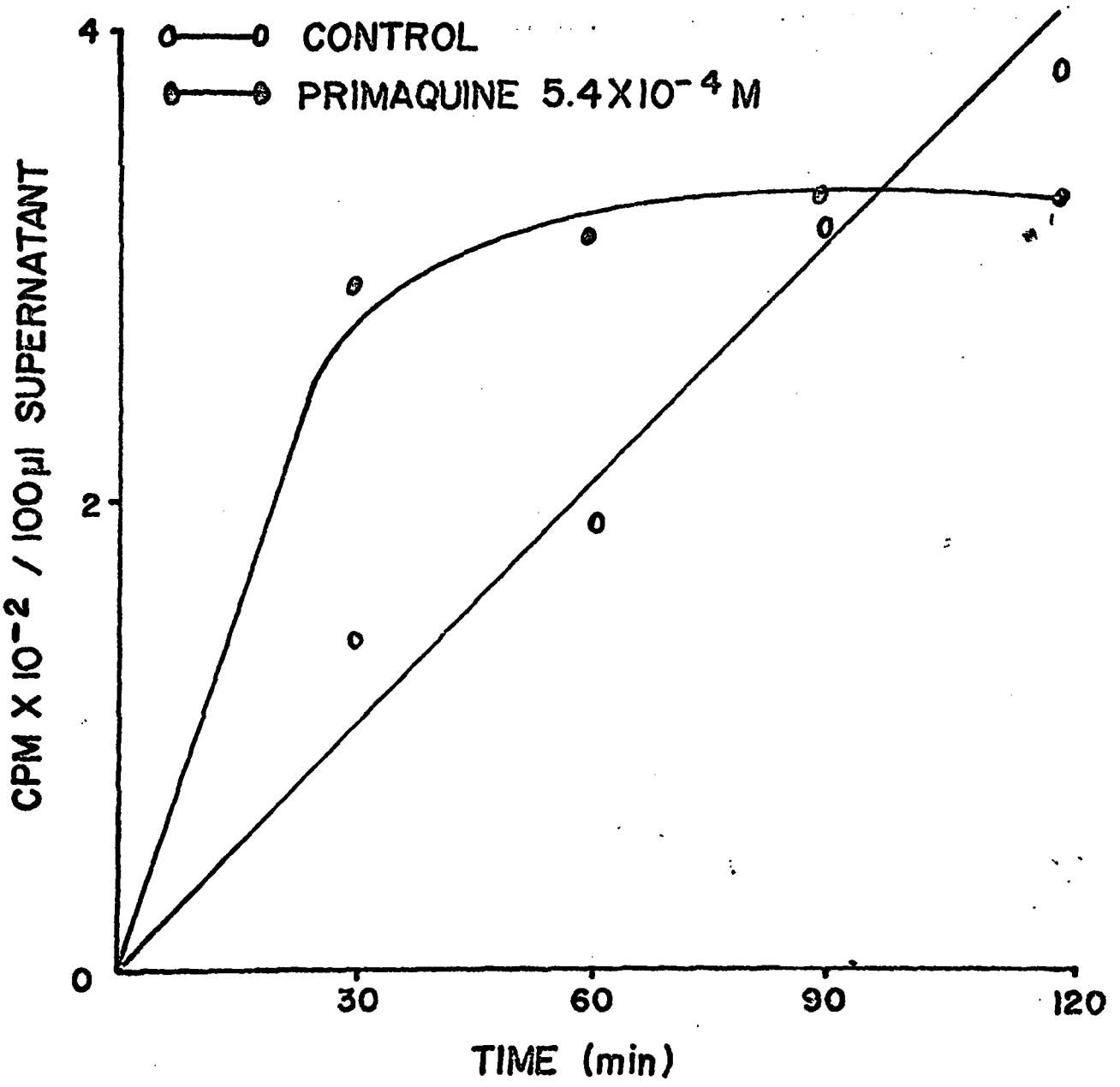


Figure 23. The effect of primaquine on uptake of ^{14}C-2-deoxy-D-glucose. The conditions are as described in legend to figure 8.

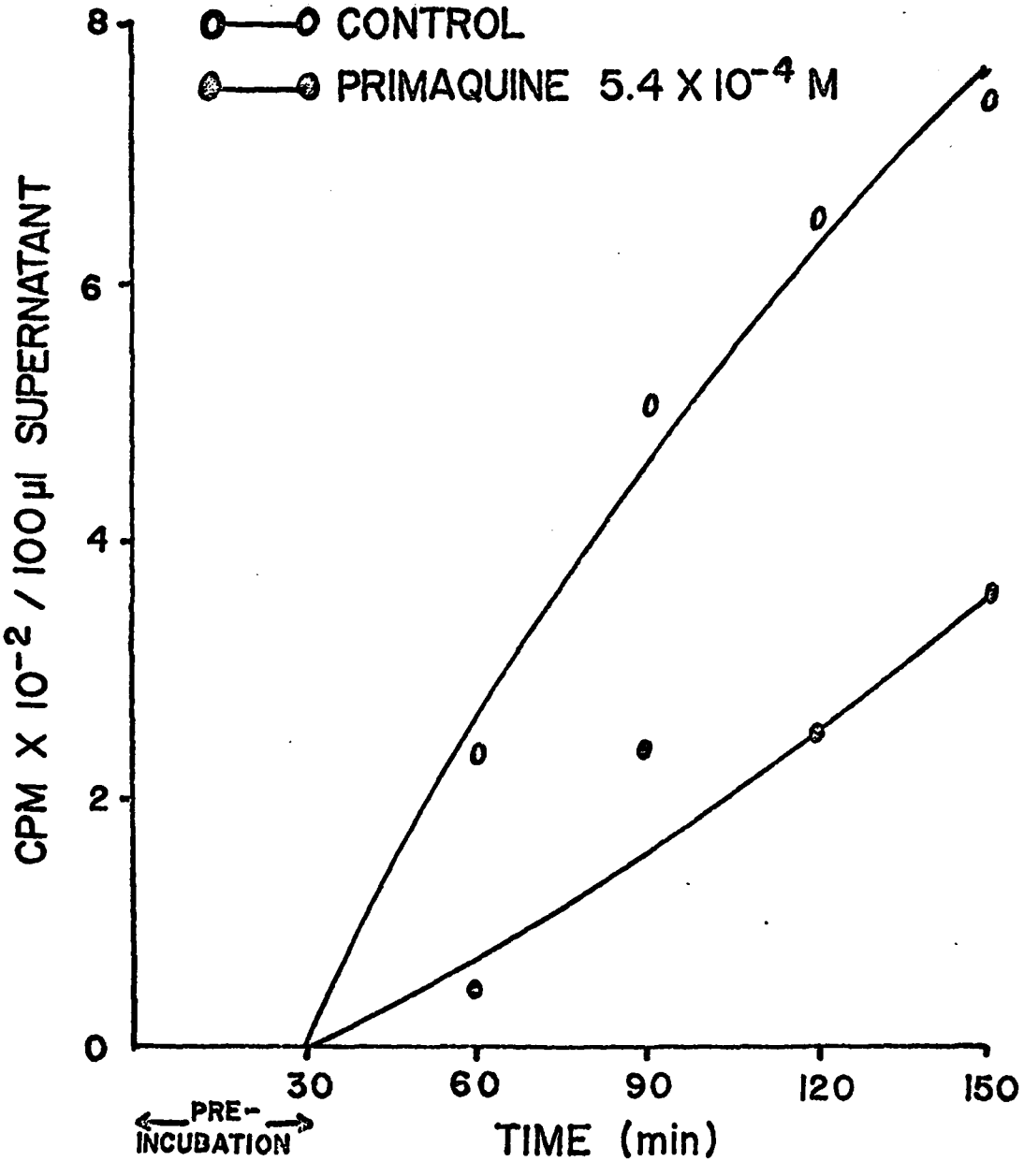


Figure 24. The effect of primaquine on uptake of ^{14}C-cycloleucine following 30 min preincubation with the drug. The conditions are as described in legend to figure 8 except that samples were taken at 30, 60, 90, 120 and 150 min.

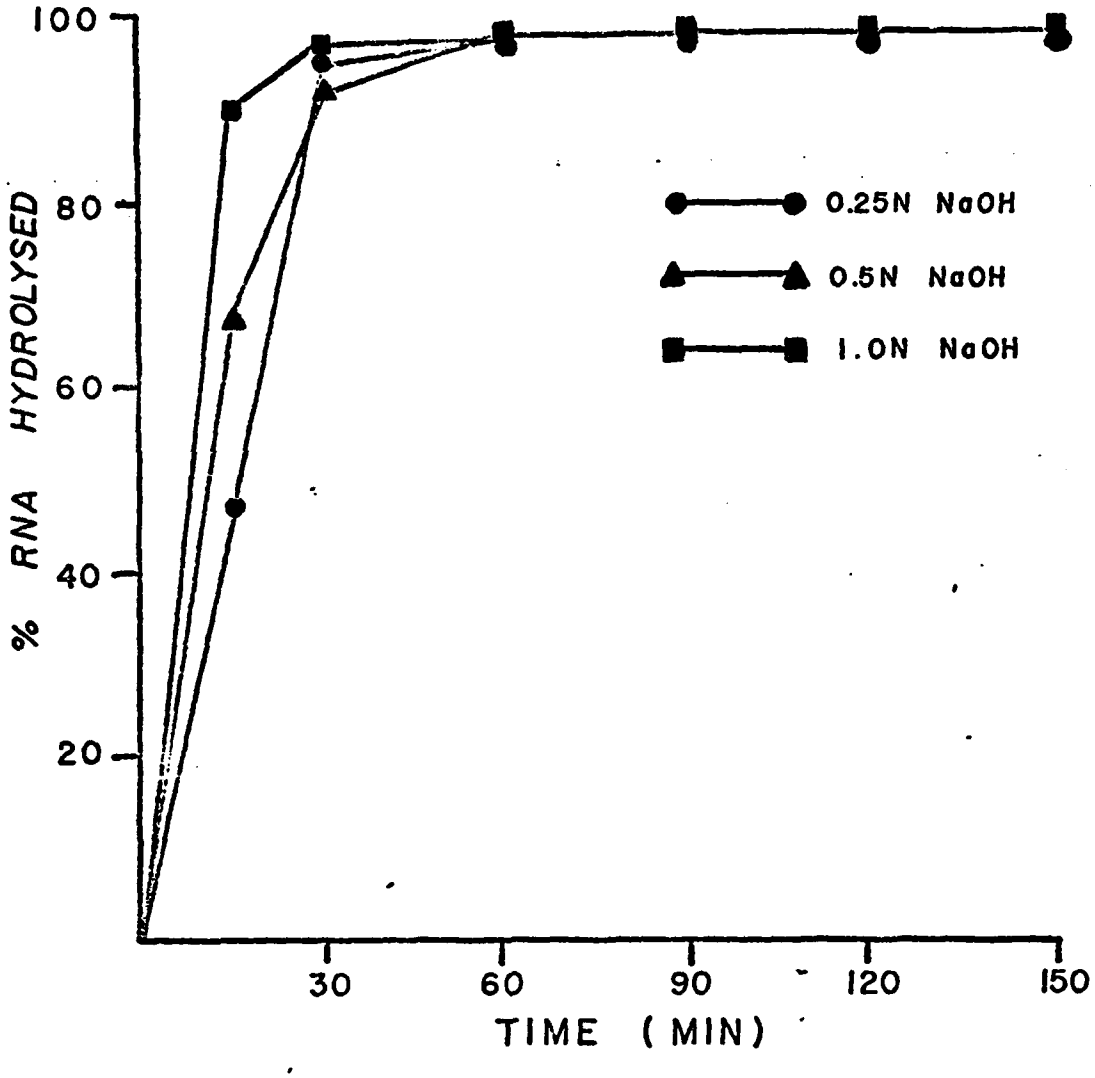


Figure 25. Time course of RNA (prepared by pulse-label) hydrolysis by different concentrations of NaOH

Cells were pulse labeled for 10 min with ^{14}C-uridine, aliquots of 0.1 ml placed on filter paper discs, and the discs processed by the filter paper disc washing procedure. The discs were impaled on pins and placed in a humidity chamber, each disc with 0.2 ml of NaOH at the indicated concentration. Discs were removed at the indicated times and processed by the filter paper disc washing procedure.

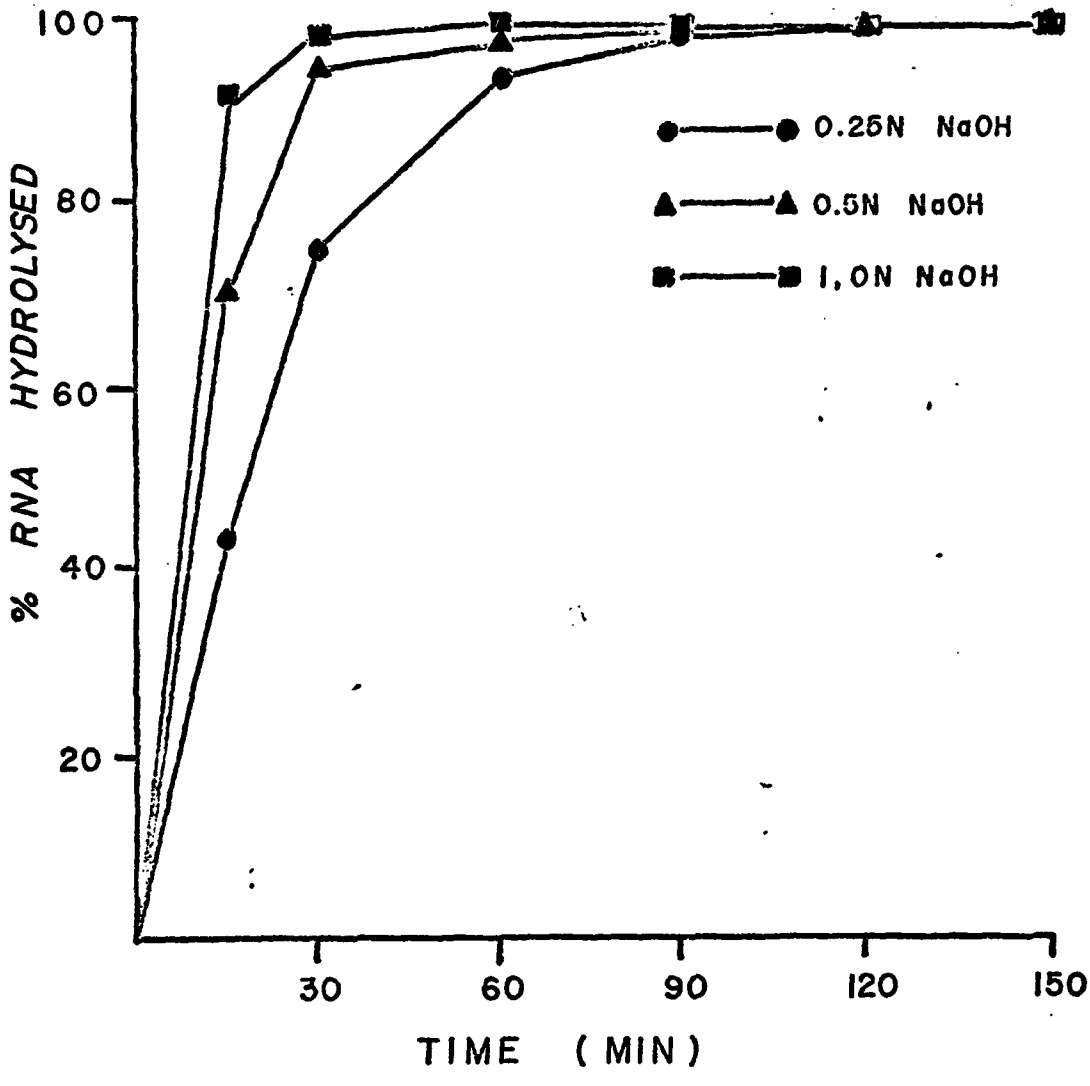


Figure 26. Time course of RNA (isolated from labeled cells) hydrolysis by different concentrations of NaOH.

Cells were grown in the presence of ^{14}C-uridine for 3 hours, followe . by extraction of the RNA by partition in a two phase polymer system of polyethylene glycol and dextran. Hydrolysis procedures are as described in Figure 7.

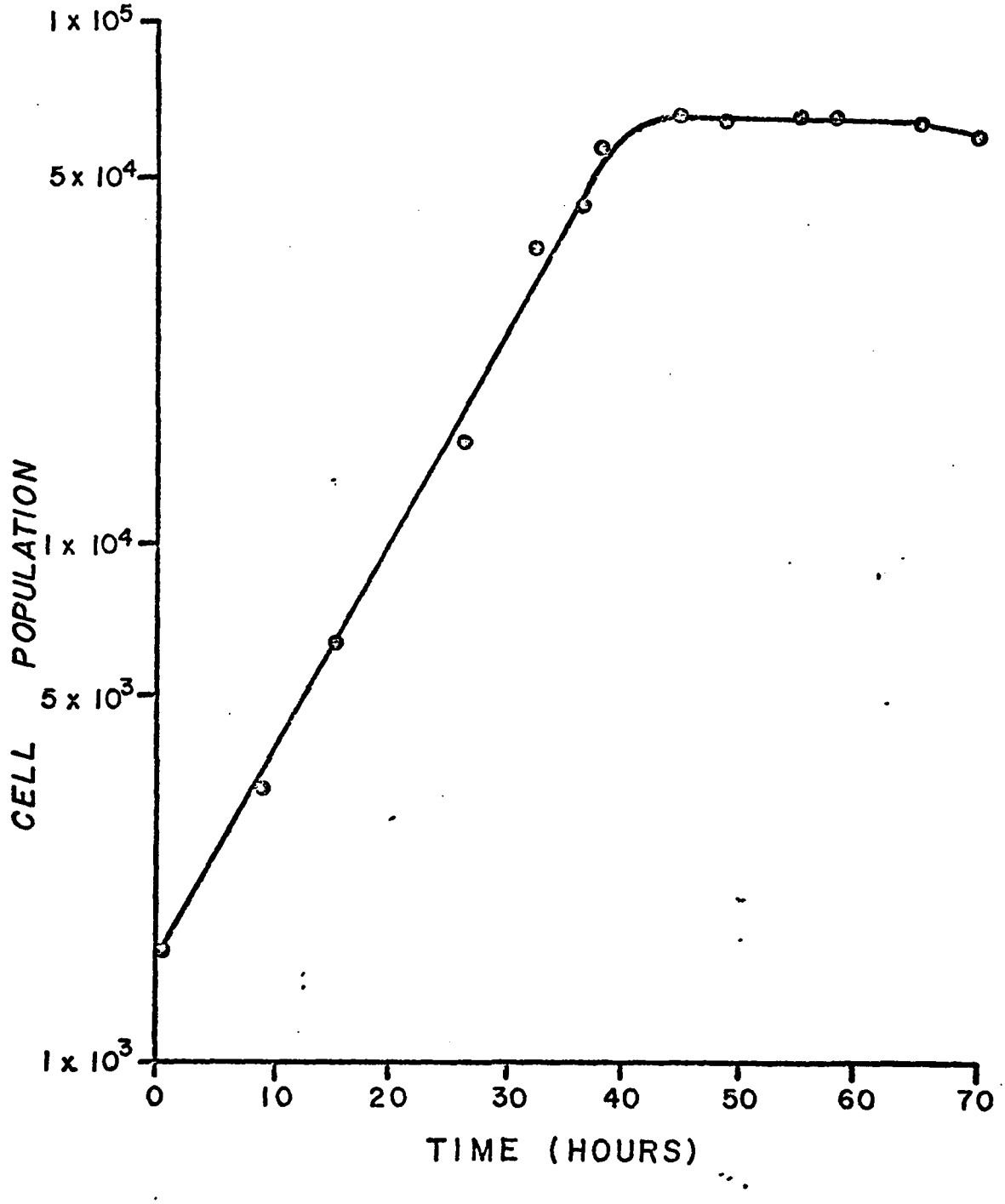


Figure 27. Growth curve for the primaquine-resistant strain of Tetrahymena pyriformis.

Cells from a stock culture of primaquine-resistant T. pyriformis were inoculated into 100 ml of 2% proteose-peptone- 0.1% liver extract medium containing 2 mg/ml primaquine. Samples were withdrawn, fixed in Ryley's buffer containing 2.5% formalin, and counted.

TABLE 1

Inhibition of synchronized cell division by primaquine[a]

Primaquine Concentration (moles/liter)	% Inhibition
1.8×10^{-4}	47
2.7×10^{-4}	89
5.4×10^{-4}	100

[a]The drug was added at EHT and cell counts were done at 0, 80, and 120 min. The percent inhibition of synchronized cell division is that which was observed at EHT plus 120 min. The initial cell population was 23,500/ml. Each value is the mean of 2 determinations.

TABLE 2

The effects of primaquine and nalidixic acid on uptake and incorporation of ^{14}C-thymidine[a]

Addition	Concentration (moles/liter)	% Inhibition (mean ± S.E.)	
		Uptake	Incorporation
Primaquine	2.7×10^{-4}	61.6 ± 0.8	67.9 ± 2.6
Nalidixic acid	8.5×10^{-4}	33.2 ± 2.1	51.7 ± 4.2

[a]The drugs and radioactive precursor were added at EHT and 0.1 ml samples were removed at 0 and 80 min. and processed by the filter paper disc procedure to determine incorporation. The percent inhibition of uptake was determined at 80 min. after EHT by sedimenting the cells and counting the radioactivity of 0.1 ml aliquots of the supernatants which had been placed on filter paper discs and dried. The cell population was 100,000/ml. Each value is from 4 determinations.

TABLE 3

The effects of primaquine and actinomycin D on uptake and incorporation of ^{14}C-uridine[a]

Addition	Concentration (moles/liter)	% Inhibition (mean ± S.E.)	
		Uptake	Incorporation
Primaquine	2.7×10^{-4}	82.5 ± 0.8	87.5 ± 0.4
Actinomycin D	4.0×10^{-5}	42.6 ± 0.7	85.4 ± 0.7

[a]The conditions are as described for Table 2.

TABLE 4

The effects of primaquine and cycloheximide on uptake and incorporation of ^{14}C-amino acids[a]

Addition	Concentration (moles/liter)	% Inhibition (mean ± S.E.)	
		Uptake	Incorporation
Primaquine	2.7×10^{-4}	57.8 ± 1.6	57.7 ± 4.4[b]
Cycloheximide	5.0×10^{-6}	48.9 ± 0.9	95.5 ± 0.7

[a]The conditions are as described for Table 2.

[b]P for uptake versus incorporation >0.05.

TABLE 5

Uptake and incorporation of precursors after 20 min. incubation with nalidixic acid actinomycin D, or cycloheximide[a]

Addition	Precursor	cpm/0.1 ml	
		Uptake	Incorporation
Nalidixic Acid	^{14}C-Thymidine	1570	140
Actinomycin D	^{14}C-Uridine	1940	186
Cycloheximide	^{14}C-Amino Acids	4055	231

[a]The drugs were added at EHT and the cells were incubated for 20 min. before adding the appropriate radioactive precursors. The cultures were incubated for an additional 60 min. Incorporation was determined as described in Figure 1, and uptake was determined as described in Figure 4. The cell populations and drug concentrations are as described in Figures 4, 5, and 6 for nalidixic acid, actinomycin D, and cycloheximide respectively.

TABLE 6

The effects of supernatant and nicotinamide on transfer RNA methylase activity of Tetrahymena pyriformis[a]

Addition	Concentration	Specific Activity[b]
None		340
Nicotinamide	5.0×10^{-3} M	340
	1.0×10^{-2} M	345
	2.5×10^{-2} M	330
Supernatant	0.6 mg/ml[c]	314
	1.2 mg/ml[c]	276
	1.8 mg/ml[c]	270

[a]The enzymes were prepared from cells in exponential growth phase.

[b]Picomoles ^{14}C-methyl transferred/hr/mg protein. Each value is the average of 3 determinations.

[c]Supernatant protein in mg/ml reaction mixture.

TABLE 7

Requirements for DNA Synthesis in Isolated Nuclei

Incubation Mixture	Counts/min./10^6 nuclei	Relative Incorporation
Standard	702	100
Minus ATP	250	36
Minus dATP	428	61
Minus dGTP	260	37
Minus dCTP	253	36
Minus dATP, dGTP, and dCTP	102	15
Minus ATP, dATP, dGTP, and dCTP	35	5
Minus EDTA	477	68
Minus KCl	568	81
Plus DNase I, 0.1 mg/ml	180	27
Plus DNase I, 0.1 mg/ml[a]	56	8
Plus RNase A, 0.1 mg/ml	912	130
Plus RNase A, 0.1 mg/ml[a]	540	78

[a]The nuclei were preincubated with the enzyme for 10 min. prior to addition to the reaction mixture.

TABLE 8

Requirements for RNA Synthesis in Isolated Nuclei

Incubation Mixture	Counts/min./10^6 nuclei	Relative Incorporation
Standard	12,690	100
Minus ATP	252	1
Minus GTP	2,360	19
Minus CTP	2,090	16
Minus ATP, GTP, and CTP	50	0
Minus NaCl and KCl	4,115	32
Plus Actinomycin D, 1.6 X 10^{-5} M	2,380	19
Plus Actinomycin D, 1.6 X 10^{-6} M	3,335	26
Plus DNase I, 0.1 mg/ml	2,740	22
Plus DNase I, 0.1 mg/ml[a]	0	0
Plus RNase A, 0.1 mg/ml	0	0
Plus RNase A, 0.1 mg/ml[a]	0	0

[a]The nuclei were preincubated with the enzyme for 10 min. prior to addition to the reaction mixture.

TABLE 9

The Effects of Primaquine on DNA Synthesis in Isolated Nuclei

Addition	Concentration (moles/liter)	Counts/min./10^6 nuclei	% Inhibition
None		748	
Primaquine	2.7×10^{-4}	612	18
	5.4×10^{-4}	556	26
	2.7×10^{-3}	74	90

TABLE 10

The Effects of Primaquine on RNA Synthesis in Isolated Nuclei

Addition	Concentration (moles/liter)	Counts/min./10^6 nuclei	% Inhibition
None		12,690	
Primaquine	2.7×10^{-4}	11,800	7
	5.4×10^{-4}	10,910	14
	2.7×10^{-3}	6,215	51

TABLE 11

Requirements for the DNA polymerase reaction by the enzyme solubilized from nuclei

One hundred µl of the pooled fractions from the Sephadex G-100 column (fractions 15-18 of Figure 1) were used in each reaction mixture. The complete reaction mixture is that described in the text for DNA synthesis by the solubilized enzyme. Each value is the mean of 2 determinations.

Reaction Mixture	cpm/µg protein	Relative Incorporation
Complete	644	100
Minus ATP	612	95
Minus dATP	386	60
Minus dCTP	245	38
Minus dGTP	335	52
Minus ATP, dATP, dCTP, dGTP	116	18
Minus KCl	193	30
Minus native DNA	13	2
Minus native DNA, plus heat-denatured DNA (50 µg)	270	42

TABLE 12

Requirements for the RNA polymerase reaction by the enzyme solubilized from nuclei

One hundred µl of the pooled fractions from the Sephadex G-100 column (fractions 15-18 of Figure 1) were used in each reaction mixture. The complete reaction mixture is that described in the text for RNA synthesis by the solubilized enzyme. Each value is the mean of 2 determinations.

Reaction mixture	cpm/µg protein	Relative Incorporation
Complete	1200	100
Minus ATP	24	2
Minus CTP	480	40
Minus GTP	384	32
Minus ATP, CTP, GTP	3	0
Minus Mn^{++}	817	68
Minus native DNA	7	0
Minus native DNA, plus heat-denatured DNA (50 µg)	889	74
Plus actinomycin D, 16 µM	468	39
Plus actinomycin D, 1.6 µM	912	76

TABLE 13

The effects of primaquine on DNA and RNA syntheses by the solubilized DNA and RNA polymerases from nuclei

Assay conditions are as described in the text with primaquine being added to the complete reaction mixtures for DNA and RNA syntheses by the solubilized polymerases. Each value is the mean of 2 determinations.

Primaquine Concentration (mM)	DNA synthesis		RNA synthesis	
	cpm/µg protein	% inhibition	cpm/µg protein	% inhibition
	600		1192	
0.27	624	0	931	22
0.54	695	0	895	25

TABLE 14

REQUIREMENTS FOR ACETATE INCORPORATION INTO LIPIDS IN THE CRUDE HOMOGENATE

Incubation Mixture	Incorporation (CPM/100 µg protein)	Relative Incorporation (%)
Complete	4620	100
Minus ATP	43	1
Minus CoA	2570	56
Minus NADH	1780	39
NADPH 2.5 mM	4080	88
NADPH 10.0 mM	3810	83
Minus Mg^{++}	3060	65
Minus EDTA	4580	99

The complete incubation mixture contained 0.1 M phosphate buffer (pH 7.2), 0.25 M sucrose, 5 mM mercaptoethanol, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 5 mM ATP, 2.5 mM NADH, 0.75 mM CoA, 0.5 µCi sodium acetate and 750 µg protein in a total volume of 0.5 ml. Samples were incubated at 29°C and 0.1 ml aliquots in duplicates were removed at 0 and 60 min, and acetate incorporation into lipids were determined by the filter paper disc procedure.

TABLE 15

EFFECT OF PRIMAQUINE ON ACETATE
INCORPORATION INTO LIPIDS IN THE CRUDE HOMOGENATE

Drug	Concentration (moles/liter)	Incorporation (CPM/100 µg protein)	Inhibition (%)
Control		4635	
Primaquine	1.1×10^{-4}	4045	12.7
	2.7×10^{-4}	3918	15.5
	5.4×10^{-4}	3500	24.5
	1.1×10^{-3}	3105	33.0

The complete incubation mixture as described in Table 14 was used and primaquine was added at the indicated concentrations. Incubation was at 29°C for 60 min and acetate incorporation was determined by the filter paper disc procedure.

TABLE 16

EFFECTS OF PRIMAQUINE ON ACETATE INCORPORATION INTO INDIVIDUAL CLASSES OF LIPIDS IN THE CRUDE HOMOGENATE

Lipid Class	Incorporation (CPM/100 µg protein) Control	Primaquine (5.4×10^{-4} M)
Triglycerides	150	65 (57)
Free Fatty Acid	983	57 (42)
Tetrahymenol	333	230 (31)
Total neutral lipids	1465	865 (41)
Phospholipids	2525	2125 (16)

Data were obtained by scraping iodine stained spots from TLC plates and counting radioactivity by scintillation spectrometry. The solvent system was chloroform : acetic acid : methanol : water (75:25:5:2.2 v/v).

*The percent inhibition is in parenthesis following each value.

TABLE 17

EFFECTS OF PRIMAQUINE ON ACETATE INCORPORATION INTO INDIVIDUAL CLASSES OF PHOSPHOLIPIDS IN THE CRUDE HOMOGENATE

Lipid Class	Control	Incorporation* (CPM/100 µg protein) Primaquine (5.4×10^{-4}M)
Cardiolipin	2223	1810 (18)
2-aminophosphonolipids	113	103 (9)
Ethanolamine Phosphatides	243	175 (28)
Choline Phosphatides	130	83 (37)
Total phospholipids	2700	2170 (20)
Total neutral lipids	1445	930 (36)
Unidentified Products	40	45

Data were obtained from scraping iodine stained spots from TLC plates and counting radioactivity by scintillation spectrometry. The solvent system was petroleum ether : diethyl ether : acetic acid (70:30:1 v/v).

*The percent inhibition is in parentheses following each value.

TABLE 18

RELATIVE ACTIVITY OF ENZYME MARKERS TO ACCESS THE PURITY OF THE MITOCHONDRIAL PREPARATION

Fraction	Description	Relative Activity (%) Succinic Dehydrogenase	Acid Phosphatase	Isocitrate Dehydrogenase
I	Pellet after first 7000 x g centrifugation	100	100	100
II	Supernatant after second 1000 x g centrifugation	42	11	26
III	Pellet after second 7000 x g centrifugation	38	9	7
IV	Final pellet (after third 7000 x g centrifugation)	36	5	3

TABLE 19

REQUIREMENTS FOR ACETATE INCORPORATION INTO LIPIDS IN THE MITOCHONDRIAL FRACTION

Incubation Mixture	Incorporation (CPM/100 µg protein)	Relative Incorporation (%)
Complete	6350	100
Minus ATP	180	3
Minus CoA	230	4
Minus NADH	2210	35
Minus NADPH	5080	89
Minus Mg^{++}	560	9
Mn^{++} 2.5 mM	5410	85
Mn^{++} 5.0 mM	4250	67
Minus EDTA	5960	94

The complete incubation mixture is as described in Material and Methods. Samples were incubated at 29°C and 0.1 ml aliquots in duplicate were removed at 0 and 30 min, and acetate incorporation into lipids was determined by the filter paper disc procedure.

TABLE 20

EFFECT OF PRIMAQUINE ON ACETATE INCORPORATION INTO LIPIDS IN THE MITOCHONDRIAL FRACTION

	Concentration (moles/liter)	Incorporation (CPM/100 µg protein)	Inhibition (%)
Control	-	6470	-
	1.1×10^{-4}	6180	4.5
	2.7×10^{-4}	5970	6.7
	5.4×10^{-4}	4390	32.1
	1.1×10^{-3}	2880	55.5

The complete incubation mixture is as described in Materials and Methods and primaquine was added at the indicated concentrations. Incubation was at 29°C for 30 min and acetate incorporation into lipids was determined by the filter paper disc procedure.

TABLE 21

EFFECT OF PRIMAQUINE ON ACETATE INCORPORATION INTO INDIVIDUAL CLASSES OF LIPIDS IN THE MITOCHONDRIAL FRACTION

Lipid Class	Incorporation* (CPM/100 g protein) Control	Primaquine (5.4×10^{-4} M)
Triglycerides	24	26 (0)
Free Fatty Acids	156	116 (26)
Tetrahymenol	1260	678 (46)
Total Neutral Lipids	1440	820 (43)
Phospholipids	3839	2510 (35)
Total Lipids	5279	3329 (37)

The separation and identification of the various classes of lipids are as described in the legend to Table 16.

*The percent inhibition is in parentheses following each value.

TABLE 22

EFFECT OF PRIMAQUINE ON INCORPORATION OF ACETATE INTO INDIVIDUAL CLASSES OF PHOSPHOLIPIDS IN THE MITOCHONDRIAL FRACTION

Lipids	Incorporation* (CPM/100 µg protein) Control	Primaquine (5.4×10^{-4} M)
Cardiolipin	480	292 (39)
2-aminophosphonolipids	524	449 (14)
Ethanolamine Phosphatides	3152	2054 (35)
Choline Phosphatides	132	92 (30)
Total Phospholipids	4288	2889 (33)
Total Neutral Lipids	1200	665 (45)
Unidentified Products	132	18 (-)
Total Lipids	5620	3575 (36)

The separation and identification of the various classes of lipids are as described in the legend to Table 17.

*The percent inhibition is in parentheses following each value.

TABLE 23

SUCCINIC DEHYDROGENASE ACTIVITY IN THE MICROSOMAL PREPARATION TO IDENTIFY CONTAMINATION WITH MITOCHONDRIA

Fraction	Description	Relative Activity of Succinic Dehydrogenase (%)
I	Supernatant before 1000 x g centrifugation	100
II	Supernatant after 1000 x g centrifugation	48
III	Supernatant after 10,000 x g	3
IV	Pellet after 105,000 x g	2

TABLE 24

REQUIREMENT FOR INCORPORATION OF ACETATE INTO LIPIDS IN THE MICROSOMAL FRACTION

Reaction Mixture	Incorporation (CPM/100 µg protein)	Relative Incorporation (%)
Complete	990	100
Minus ATP	8	1
Minus CoA	8	1
Minus NADH	220	22
NADPH 0.8	960	97
2.0	820	83
4.0	650	66
Minus Mg^{++}	390	39
Mn^{++} 5 mM	740	75
10 mM	610	62
EDTA 0.1 mM	960	97
0.25 mM	910	92

The complete incubation mixture is as described in Materials and Methods. Samples were incubated at 29°C and 0.1 ml aliquots in duplicates were removed at 0 and 30 min, and acetate incorporation into lipids were determined by the filter paper disc procedure.

TABLE 25

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO LIPIDS IN THE MICROSOMAL FRACTION

	Concentration (moles/liter)	Incorporation (CPM/100 µg protein)	Inhibition (%)
Control		1000	-
Primaquine	1.1×10^{-4}	920	8
	2.7×10^{-4}	710	29
	5.4×10^{-4}	600	40
	1.1×10^{-3}	480	52

The complete reaction mixture is as described in Materials and Methods and primaquine was added at the indicated concentrations. Incubations was at 29°C for 30 min and acetate incorporation into lipids was determined by the filter paper disc procedure.

TABLE 26

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO THE CLASSES OF LIPIDS SYNTHESIZED BY THE MICROSOMAL FRACTION

Lipid	Incorporation* (CPM/100 µg protein) Control	Primaquine (5.4×10^{-4} M)
Triglycerides	38	15 (60.5)
Free Fatty Acids	435	196 (54.9)
Tetrahymenol	52	20 (61.5)
Total Neutral Lipids	525	231 (56.0)
Phospholipids	225	249 (0)
Total Lipids	750	480 (36)

The separation and identification of the various classes of lipids are described in the legend to Table 16.

*The percent inhibition is in parentheses following each value.

TABLE 27

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO THE CLASSES OF PHOSPHOLIPIDS SYNTHESIZED BY THE MICROSOMAL FRACTION

Lipid	Control	Primaquine (5.4×10^{-4} M)
Cardiolipin	8	10 (0)
2-aminophosphonolipids	30	30 (0)
Ethanolamine Phosphatides	165	170 (0)
Choline Phosphatides	2	8 (0)
Total Phospholipids	205	218 (0)
Total Neutral Lipids	480	230 (52.0)
Unidentified	15	-
Total	700	448 (36)

The separation and identification of the various classes of lipids are as described in the legend to Table 17.

*The percent inhibition is in parentheses following each value.

TABLE 28

REQUIREMENTS FOR THE INCORPORATION OF ACETATE INTO LIPIDS IN THE SOLUBLE CELL FRACTION

Reaction Mixture	Incorporation (CPM/100 µg protein)	Relative Incorporation (%)
Complete	2610	100
Minus ATP	30	1
Minus CoA	70	3
Minus NADH	120	5
Minus NADPH	120	5
Minus Mg^{++}	2480	95
Minus Mn^{++}	1530	59
Minus EDTA	1780	68

The complete incubation mixture is as described in Materials and Methods. Samples were incubated at 29°C and 0.1 ml aliquots in duplicate were removed at 0 and 90 min, and acetate incorporation into lipids was determined by the filter paper disc procedure.

TABLE 29

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO LIPIDS SYNTHESIZED BY THE SOLUBLE CELL FRACTION

	Concentration (moles/liter)	Incorporation (CPM/100 µg protein)	Inhibition (%)
Control	-	2620	-
Primaquine	1.1×10^{-4}	2020	22.9
	2.7×10^{-4}	1550	42.8
	5.4×10^{-4}	980	62.5
	1.1×10^{-4}	390	85.2

The complete incubation mixture is as described in Material and Methods. Incubation was at 29°C for 90 min and acetate incorporation into lipids was determined by the filter paper disc procedure.

TABLE 30

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO THE CLASSES OF LIPIDS SYNTHESIZED BY THE SOLUBLE CELL FRACTION

	Incorporation* (CPM/100 µg protein)	
	Control	Primaquine (5.4×10^{-4}M)
Triglycerides	109	40 (63.3)
Free Fatty Acids	620	80 (87.0)
Tetrahymenol	745	300 (59.7)
Total Neutral Lipids	1474	420 (71.5)
Phospholipids	746	576 (22.8)
Total Lipids	2200	960 (56.4)

The separation and identification of the various classes of lipids are as described in the legend to Table 16.

*The percent inhibition is in parentheses following each value.

TABLE 31

EFFECT OF PRIMAQUINE ON THE INCORPORATION OF ACETATE INTO THE CLASSES OF PHOSPHOLIPIDS SYNTHESIZED BY THE SOLUBLE CELL FRACTION

Lipid	Incorporation* (CPM/100 µg protein) Control	Primaquine (5.4×10^{-4}M)
Cardiolipin	-	4 (-)
2-aminophosphonolipids	240	140 (41.7)
Ethanolamine Phosphatides	321	266 (17.1)
Choline Phosphatides	172	70 (59.3)
Total Phospholipids	734	580 (21.0)
Total Neutral Lipids	1466	376 (74.4)
Unidentified	40	24 (40)
Total	2240	980 (56.2)

The separation and identification of the various classes of lipids are as described in the legend to Table 17.

*The percent inhibition is in parentheses following each value.

TABLE 32

Hydrolysis of DNA on filter paper discs by hot trichloroacetic acid

The filter paper discs were prepared from 0.1 ml aliquots of cells pulse-labeled with ^{14}C-thymidine (pulse-labeled DNA) or 0.1 ml of a solution containing DNA extracted by partition in a two phase polymer system from cells labeled for 3 hours with ^{14}C-thymidine (Isolated DNA). The discs were processed by the filter paper disc washing procedure, followed by treatment with the NaOH hydrolysis procedure for RNA. The resulting discs were then treated for the indicated times with 5% TCA at 90°C.

Time (Min)	% Hydrolysis	
	Pulse-labeled DNA	Isolated DNA
15	95	99
30	96	99
45	96	99

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